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The characteristic of sleep observed during infectious disease or after the administration of certain bacterial products or endogenous cytokines is similar to the sleep observed after sleep deprivation. Such results have led to the hypothesis that cytokines and perhaps certain bacterial products are involved in physiological sleep regulation; such substances are termed sleep factors (SFs). Our investigations have led us to propose a biochemical regulatory scheme for sleep in which the somnogenic actions of many putative SFs are linked to each other in the sense that they alter each other's production and/or activity. Over the past four years of this contract, we have described several additional SFs and interconnections between them. These results

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indicate that new more effective and safer somnogenic agents can be developed. The broad goal of our USAMRDC-supported worked has been to develop the information needed to ascertain if it is reasonable to propose either endogenous SFs or synthetic analogs as potential sleep-inducing agents. To meet this goal in previous years we reported our progress on 16 sets of experiments; this year the results from an additional 5 sets of experiments are reported. Briefly, results are as follows: 1) We showed that interleukin-1 α and as a synthetic interleukin-1 β fragment are somnogenic; a patent on the latter substance is in the process of being prepared. 2) We also designed another synthetic peptide based on the complementary peptide theory. Thus, a peptide complementary to growth hormone releasing hormone, (i.e. its sequence was derived from the negative sense mRNA) was found to be somnogenic. A patent for this substance has been submitted. 3) We also extended our observations on the sleep disturbances occurring during infectious disease by showing that the time courses of sleep, body temperature and changes in several blood parameters depends on the localization of the infection. 4) Our fourth experiment expanded upon the third by showing that bacterial peptidoglycan whether obtained from gram negative or from gram positive bacteria was somnogenic. 5) A mechanism critical to the changes in sleep observed during infection or after peptidoglycan was also investigated. We showed that mammalian macrophages could digest bacterial cell walls to produce somnogenic muramyl peptides. These results are discussed with the context of a new theory for brain organization for sleep. On a more practical level, it seems likely that these results are leading to the development of one or more new somnogenic agents.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW Publication No. (NIH) 78-23, Revised 1978].

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ABBREVIATIONS

A	absorbance
aCSF	artificial cerebrospinal fluid
CFU	colony-forming unit
CP	complementary peptide
DNA	delta wave amplitudes
EEG	electroencephalographic
GH	growth hormone
hGHRH	human growth hormone releasing hormones
HPLC	high pressure liquid chromatography
ICV	intracerebroventricular
IL1	interleukin-1
im	intramuscular
in	intranasal
iv	intravenous
LAL	limulus amoebocyte lysate
LPS	lipopolysaccharide
MDP	muramyl dipeptide
MEM	minimal essential medium
MP	muramyl peptide
nRBC	nucleated red blood cells
NREMS	non-rapid-eye-movement sleep
PFS	pyrogen-free saline
PG	peptidoglycan
PGD_2	prostaglandin D_2
RA	rheumatoid arthritis
REMS	rapid-eye-movement sleep
sc	subcutaneous
SF	sleep factor
SWS	slow-wave sleep
T_{br}	brain temperature
T_{co}	colonic temperature
TFA	trifluoroacetic acid
TNF	tumor necrosis factor
W	wakefulness
WBC	white blood cells

INTRODUCTION

The primary evidence that sleep is regulated by homeostatic mechanisms is the demonstration that if one is deprived of sleep, then allowed to sleep there is a rebound (excess) of sleep. In man and animals, this excess sleep continues to be episodic and maintains elements of circadian organization. It also seems to be of greater intensity than normal sleep in the sense that arousal thresholds are higher (it's harder to wake a person from sleep after prolonged sleep deprivation). Further, the amplitudes of electroencephalographic (EEG) slow waves during slow-wave sleep (SWS) are higher after sleep deprivation than during normal sleep (104). As will be discussed, these characteristics of sleep are also observed during infectious disease or after administration of cytokines and microbial products. These latter substances have been implicated in sleep regulation and are thus, termed sleep factors (SFs).

Research focused on identifying SFs began at the turn of the century when Legendre and Pieron and Ishimori (reviewed, 71) described the accumulation of SFs in cerebrospinal fluid (CSF) during prolonged wakefulness (W). Over the next 80 years, about 30 putative SFs have been identified; many of these are hormones [e.g., growth hormone releasing factor (102)] or are related to the hosts' response to infectious disease [e.g., interleukin-1 (73)]. Our investigations have led us to propose a biochemical regulatory scheme for sleep in which it is illustrated that the somnogenic actions of most of the putative SFs are linked to each

other in the sense that they can alter each other's production and/or activity. Over the past four years of this contract, we have described several additional SFs and interactions between them (71). These results indicate to us that new, more effective and safer somnogenic agents can be developed. The primary objective of our USAMRDC-supported work has been to develop the information needed to ascertain if it is reasonable to propose either endogenous SFs or synthetic analogs as potential sleep-inducing agents. Towards this end, this year we have initiated the process to obtain a patent on one of the synthetic SFs we discovered.

With the above objective in mind, in the first year of our contract period (June 1, 1986 - May 30, 1987), five sets of experiments were performed; in the second year (June 1, 1987 - May 30, 1988), six sets of experiments were performed and in the third year (June 1, 1988 - May 30, 1989), five set of experiments were performed. Results from those experiments are described in our annual reports dated June 1, 1987, June 1, 1988 and June 1, 1989. This year (June 1, 1989 - May 30, 1990), an additional five sets of experiments were performed; they are numbered one through five as follows:

1) In our first experiment, we show that a synthetic fragment of interleukin-1β (IL1β) has somnogenic activity.
2) Second, we designed and synthesized a peptide complementary to GRH that was also somnogenic.

3) In our third experiment, we extended our observations concerning infectious disease and sleep by showing that sleep disturbances occurring during infection depend upon the route of administration.

4) Our fourth experiment expanded upon the third by showing that the increase in sleep associated with infection can be elicited by isolated bacterial cell walls.

5) Finally, we showed that mammalian macrophages tailor somnogenic muramyl peptides during their digestion of bacterial cell walls.

METHODS

Animals. A lateral cerebral ventricular guide cannula, a thermistor, and stainless steel screw electroencephalographic (EEG) electrodes were surgically implanted into male New Zealand White Pasteurella-free rabbits (about 3.5 kg), as previously described (73, 121). EEG electrodes were placed over the frontal and parietal cortices. A 50,000-ohm calibrated thermistor (model 4018, Omega Engineering, Stamford, CT) was implanted over the parietal cortex to measure brain temperature (T_{br}). Insulated leads from the screws and the thermistor were routed to an Amphenol plug attached to the skull with dental acrylic (Duz-All, Coralite Dental Products, Skokie, IL). The guide cannula was placed in the left lateral cerebral ventricle. A minimum of two weeks was allowed for recovery before the animals were habituated to the recording chambers.

The rabbits were housed in an animal facility on a 12:12 h light-dark cycle (lights on, 0600) at 21°C. Prior to experimentation, the animals were habituated to the recording chambers (Hotpack 352600, Philadelphia, PA) for at least two 24-h sessions. When experiments were scheduled, the rabbits were placed in the recording chambers the preceding evening, with the light-dark and temperature regimens of the recording chambers maintained under the same conditions as the animal facility. Food and water were available ad libitum.

Apparatus and recording. Each recording chamber contained an electronic swivel (Stoelting, Chicago, IL) suspended by a shock-absorbing system. A flexible tether connected the swivel to the Amphenol connector on the rabbit's head, thus allowing freedom of movement. An accelerometer (Grass, SPA1, Quincy, MA) attached to the shock-absorbing system provided an indication of body movement. The cables from the swivel and the accelerometer were connected to Grass 7D polygraphs in an adjacent room. These polygraphs recorded EEG, T_{br}, and body movement for each animal. The EEG for each rabbit was passed through band-pass filters with the 0.5-3.5 Hz (delta), 4.0-7.5 Hz (theta), 8.0-12.5 Hz (alpha), and 13.0-25.0 Hz (beta) frequency bands rectified and averaged for 1-min intervals by a Buxco model 24/32 data logger (Buxco Electronics, Sharon, CT). These values for 1-min periods were used to compute hourly averages. EEG amplitudes in the delta frequency band during 1-min epochs of NREMS for each of the six postinjection hours were also determined. The 10 samples of greatest magnitude in each hour were averaged for each rabbit

during control and test NREMS periods. In addition, the ratio of theta to delta activity was also computed and displayed on the polygraph simultaneously with the EEG, T_{br}, and body movement to facilitate scoring of vigilance states. T_{br}s were also recorded using a data logger (Acrosystems 400, Beverly, MA), with values for each rabbit sampled at 10-min intervals. Colonic temperatures (T_{co}) were taken with a flexible thermistor (YSI, Inc., Yellow Springs, OH) at the time of injection and at the end of the 6-h recording session.

The polygraph records were visually scored in 12-s epochs for periods of SWS, REMS, and W. W is characterized by low-voltage rapid EEG, frequent body movements, midlevel EEG theta-to-delta ratios, and decreasing T_{br} following REMS or increasing T_{br} following SWS. During periods of SWS, increased EEG slow-wave voltage, decreased T_{br}, little or no body movement, and low theta-to-delta ratios are observed. REMS is identified by low-voltage EEG, a rapid rise in T_{br}, sporadic occurrence of body movements, and high theta-to-delta ratios.

Specific Experimental Protocols

Experiment No. 1: Somnogenic activity of IL1 fragments

Compounds tested. Recombinant human IL1β (hu-rIL1β) was a gift from Dr. C. Dinarello (Tufts University School of Medicine) and was prepared by Cistron Biotechnology, Inc. (Pine Brook, NJ). Recombinant human IL1α (hu-rIL1α) was prepared by Hoffman-LaRoche (Nuttley, NJ). IL1β peptide 117-134, representing the 18 N-terminal residues of biologically active human IL1β and peptide

247-269, representing the 23 C-terminal residues of human IL1β were gifts from Dr. Louis Chedid (University of South Florida). IL1α fragments 115-148 and 223-251 and IL1β fragments 178-207, 199-225, and 208-240 were synthesized by Dr. Jerome M. Seyer (Table 1).

Experimental protocol. IL1 fragments were dissolved in appropriate volumes of artificial cerebrospinal fluid (aCSF) (2 mM KCl, 1.15 mM $CaCl_2$, and 0.96 mM $MgCl_2$ in pyrogen free saline, 155 mM NaCl [Abbott, N. Chicago, IL]). Hu-rIL1β was supplied to us in phosphate-buffered saline (PBS) (0.4 mg/ml) and hu-rIL1α was supplied to us in 30 mM Tris-Cl, 0.4 M NaCl, pH 7.8 (0.5 mg protein/ml). Appropriate aliquots of IL1α (0.01-10 ng) and β (5-20 ng) were then diluted in aCSF. All substances were injected into a lateral cerebral ventricle (ICV). Injection volumes were 12-25 µl per rabbit; each injection lasted about 2 min. Animals were injected between 0900 and 1000 h. Control recordings were obtained from each animal after injection of vehicle (aCSF). An additional control for hu-rIL1α was obtained by injecting heat-inactivated (75°, 30 min) hu-rIL1α; a similar procedure inactivates IL1β somnogenic activity (73). After injections, animals were returned to their cages and then were recorded from for the next 6 h.

PGE_2 production by rheumatoid arthritis synovial cells. Synovial tissue obtained at surgery from patients with rheumatoid arthritis (RA) undergoing joint replacement was cut into pieces of 2 mm or less and incubated at approximately 100 mg/ml in serum-free minimal essential medium (MEM) containing 1 mg/ml bacterial collagenase (Sigma Chemical Co., St. Louis, MO)

and 10 µg/ml testicular hyaluronidase (Sigma) at 37°C with rocking. After 3-4 h, the suspension was centrifuged at 150 xg for 10 min. The cells were resuspended (10^5 cells/ml) in MEM containing 20% fetal calf serum (FCS) and plated in 100-mm diameter Petri dishes (10 ml per dish). The disaggregated cells were cultured overnight at 37°C in 5% CO_2, and the nonadherent cells were aspirated off by vigorous pipetting. Adherent synovial cells were maintained in culture with medium change every four days until they reached confluency, at which time they were subcultured after trypsinization and grown in maintenance medium (Eagle's MEM supplemented with nonessential amino acids, ascorbic acid, amphotericin B (1 µg/ml), $NaHCO_3$, penicillin (100 U/ml), and streptomycin (100 µg/ml), FCS (9%), and PBS.

Adherent rheumatoid synovial cells (ARSC) were set up in 24-well number 3424 Costar plates (Cambridge, MA) by adding 5 x 10^4 cells per well in 0.5 ml of maintenance medium. After 72 h, the monolayers were confluent, and medium was changed to serum-free maintenance medium for 24 h. Medium was changed, and to each well was added 450 µl serum-free maintenance medium and 50 µl PBS or 50 µl PBS containing various concentrations of synthetic IL1α or β peptides, previously sterilized by micropore filtration. After 24 h, culture supernatants were harvested, and PGE_2 was extracted from the supernatants and quantitated by radioimmunoassay as previously described (108).

T cell proliferation assays. Thymus glands were removed from 6-10 week old CD1 mice after mice had been sacrificed by cervical dislocation. Thymocytes were isolated by gently

homogenizing the thymus glands in a dounce homogenizer. Thymocytes were suspended in RPMI 1640 medium, washed once, and resuspended (10^7 cells/ml) in RPMI 1640 containing 7.5% FCS and 5 x 10^{-6} M 2-mercaptoethanol. Aliquots of the cell suspension (100 µl) were dispensed into wells of flat bottom microtiter plates (Linbro Plastics), and samples (50 µl) suspended in RPMI 1640 were added to appropriate wells. Plates were incubated at 37°C, 5% CO_2, in a humidified atmosphere for 60 h, and wells were pulsed with [^{3}H] thymidine (1 µCi, 1.9 Ci/mol) for 12 h. After the 12-h pulse, cells were harvested onto paper filter pads with a multiple-sample harvester, and thymocyte proliferation was determined by scintillation counting. In some experiments, the murine T cell line D10 was used instead of thymocytes as target cells as previously described using phytohemagglutinin (1 µg/ml) as costimulant (66).

Statistical analysis. Data from all experiments were analyzed with the SPSS^X Information System. Friedman's test for k-related samples was used to test for differences across the 6-h recording periods. If significant differences were indicated, the Wilcoxon matched-pairs signed-ranks test was used to identify the specific hours in which the differences occurred. On alpha level of $p < 0.05$ was accepted as indicating significance.

Experiment No. 2: Complementary GHRH induces sleep

Peptide. The sequence of the complementary peptide (CP) of rat (r)GHRH was derived from the hypothetical messenger RNA (mRNA) complementary to the mRNA of rGHRH (Fig. 4). The rGHRH mRNA sequence was published by Mayo et al. (95). The complementary

mRNA (-mRNA) was translated in the 3' to 5' direction, corresponding to N-terminal to C-terminal direction for the peptide sequence. The reading resulted in 4 peptides, interrupted by 3 STOP codons. When choosing from these peptides, we considered the following observations. The biological activity of human GHRH (hGHRH) is decreased to 0.1% by the deletion of the N-terminal amino acid (50). Step-by-step deletion of the C-terminal portion of hGHRH results in gradual decrease in biological activity until reaching hGHRH (1-27) NH_2 (12%). Then the bioactivity sharply decreases to 0.02% (hGHRH [1-24] OH), and hGHRH (1-19) NH_2 is completely inactive (83). Therefore, the N-terminal and the middle (20-27) regions of GHRH are involved in binding to the GHRH receptor, and the complementary sequence of these regions would be suspected to be homologous with the binding sites of the GHRH receptor. As the CP of the N-terminal region was interrupted by a STOP codon, resulting in a tetrapeptide, we focused our attention to the peptide sequence complementary to rGHRH (14-25), which is long enough to form a secondary structure based on Chou-Fasman calculations (25). We refer to this sequence as 3'-5' CP. The C-terminal amino acid (Leu) was amidated because this CP sequence is (hypothetically) only a region of a larger protein sequence, where the C-terminal Leu would be in a peptide bond.

The 3'-5' CP was synthesized at the Molecular Resource Center, Macromolecular Synthesis Laboratory, University of Tennessee, Memphis (Director: Dr. T. G. Cooper) by Dr. R. A. Sumrade, using an Applied Biosystems Model 430A fully automatic

instrument-reagent system for solid phase peptide synthesis. The peptide was removed from the resin and partially purified by Immuno-Dynamics, Inc. (La Jolla, CA). The resulting preparation was further purified using a Varian LC 5000 HPLC and a Rainin Microsorb C_{18} reversed phase column (25 cm x 4.6 mm). A Kratos Spectroflow 773 absorbance detector operated at 230 nm was used for detection. The solvent program applied was linear from 0% to 60% CH_3CN (containing 0.1% CF_3COOH) in water (containing 0.7% CF_3COOH) over a period of 30 min at a flow rate of 1 ml/min. Two elution peaks were detected; the larger peak eluted at 25.2 min and contained about 90% of the total recovered as estimated by UV absorbance. The larger peak was collected and lyophilized. Its sequence was verified by Dr. Jerome M. Seyer of the Veterans Administration Medical Center in Memphis, Tennessee, using a Beckman 890 sequencer and a Beckman 121 MB amino acid analyzer. Values obtained by amino-acid analysis were used to calculate doses used.

Pituitary cell superfusion. Pituitary glands obtained from adult male Holtzman rats (body weight: 250-300 g) were collected in Medium 199 containing 0.3% BSA, 44 mM sodium bicarbonate, 10 mM HEPES, 10 mg/ml gentamycin, 2 mg/100 ml bacitracin, pH 7.4, and were enzymatically dispersed according to the method of Loumaye and Catt (86). Briefly, the pituitaries were minced and then digested with a 5 mg/ml trypsin (1:250, Difco, Detroit, MI) solution for 15 min at 37°C with constant shaking. After removal of the trypsin solution, the digested glands were further incubated in 5 ml EDTA-bisphosphate (15mM

Na_2HPO_4/KH_2PO_4, 2 mM Na_2-EDTA, 135 mM NaCl, pH 7.4, 10 mg/ml gentamycin, 0.1% BSA) at 37°C for 5 min with constant shaking. After the tissue was triturated 20 times, 45 ml bisphosphate (without EDTA) was added, and the solution was filtered through a nylon mesh, followed by centrifugation at 1000 rpm for 10 min. The cells were further treated with 5 ml of 2 mg/ml DNase I solution (Sigma, Type IV, in the above M 199 medium) at room temperature for 2 min with gentle swirling. After centrifugation, the cells were resuspended in 10 ml of the aforementioned culture medium. For each column, 1.5 ml of the cell suspension (approximately $8x10^5$ cells) were mixed with 400 mg preswollen Bio-Gel P-2 and packed into 1.5 ml columns of an Acusyst S® perifusion system. The cells were perifused with the above culture medium supplemented with 5 nM dexamethasone (133) at 100 ml/min flow rate, under continual gassing with 95%O_2/5%CO_2. After a 3-h stabilization period, the each of the 6 columns were exposed to 3'-5' CP 3 times in the order of increasing concentrations (0.1, 1.0 and 10 nM).

Growth Hormone Assay. Immunoreactive GH was assayed using materials kindly provided by Dr. A. F. Parlow and the National Hormone and Pituitary Program, NIDDK. Rat GH was labelled with ^{125}I using chloramine-T, and the labelled hormone was purified on a Sephadex G-75 gel filtration column. The standard and the samples were incubated with the antiserum (final dilution: 1:30,000) and the tracer (approximately 16,000 cpm/tube) for 22 h at room temperature (20-22°C) in a final volume of 600 ml/tube. Bound and free hormone were separated by *Staphylococcus* protein A

(IgGsorb, The Enzyme Center, Malden, MA). The nonspecific binding is 2-5%, the specific binding of the tracer at zero concentration of the cold antigen is 40-45%. The detection limit of the assay is 30 pg/tube (in terms of rGH RP-2 standard), the maximum sample volume (serum) is 120 ml/tube, and the intra- and interassay variations at 50% displacement are <5%. IrGH was detected in duplicate 10 ml/tube of the culture medium. To avoid interassay variation, all samples were measured in a single assay. The 3'-5' CP did not cross-react with the anti-rGH antiserum.

Sleep studies in rabbits. After habituation to the recording chambers, each rabbit was injected ICV with 25 µl artificial cerebrospinal fluid (aCSF) and 1 nmol/kg 3'-5' CP dissolved in aCSF on two different days. The 3'-5' CP was tested *in vivo* before and after HPLC purification. EEG, brain temperature and motor activity were recorded for 6 hours postinjection. The percent of time spent in the individual states of vigilance (W, NREMS, REMS) was determined for each postinjection hour and for the total of the 6-h recording period.

Statistical Analyses. The hydropathic scores and secondary structure of rGHRH and the 3'-5' CP were analyzed by the methods of Kyte & Doolittle (81) and Chou & Fasman (25), respectively. The effect of the 3'-5' CP on GH secretion *in vitro* was analyzed by one-way ANOVA, followed by Newman-Keuls multiple range test, considering the last prestimulation GH level as a control. The durations of individual states of vigilance on the aCSF-days and 3'-5' CP days were compared using the Wilcoxon matched pairs signed ranks test.

Experiment No. 3: Experimental Pasteurellosis: effects on sleep

Base-line sleep patterns were monitored for 24 h before the rabbits received any experimental treatment. At 8:00 a.m. on the following day (time 0 on figures), rabbits were inoculated with *P. multocida* as described below, and recording was continued for an additional 24-48 h. Blood samples and rectal temperatures were obtained from each rabbit before microbial challenge and at 6- or 12-h intervals after inoculation. During the recording period, rabbits were able to move freely in their cages and had continuous access to food and water.

The strain of *P. multocida* used in these experiments (serotype 12:A) was generously supplied by Dr. Y. S. Lu of the University of Texas Health Science Center at Dallas and is known to be pathogenic for rabbits (87). For preparation of inocula, stock cultures of frozen organisms were incubated on blood agar plates overnight at 37°C in a 5% CO_2 atmosphere. Colonies were transferred to sterile pyrogen-free saline to achieve a concentration of ~2 x 10^9 colony-forming units (CFU)/ml. The number of CFU was initially estimated using a Klett-Summerson photoelectric colorimeter and later verified by plating serial dilutions of the bacterial suspension on blood agar plates and counting colonies after a 24-h incubation.

Rabbits were inoculated with 0.7 ml of bacterial suspension containing 6.7 ± 1.2 x 10^7 colony-forming units (CFU) for iv inoculation (n = 8), 8.2 ± 0.4 x 10^8 CFU for im inoculation (n = 6), 9.6 ± 1.2 x 10^8 CFU for intranasal inoculation (n = 10), or 2.9

± 0.2 x 10^9 CFU for sc inoculation (n = 6). The severe clinical disease that resulted from iv administration necessitated using a lower challenge dose with this route of inoculation, and for humane reasons most of these animals were maintained for only 24 h after challenge. Rabbits inoculated by im, in, and sc routes were monitored for 48 h after challenge. Each rabbit was inoculated only once and was killed at the end of the experiment with iv T61 euthanasia solution (Taylor Pharmacal, Decatur, IL). Rabbits inoculated with equal volumes of saline (n = 2 for each route of inoculation) represent the control group.

Blood samples (1-2 ml) were collected from the central artery of the ear and were immediately transferred to vacuum tubes containing EDTA (Vacutainer Systems, Rutherford, NJ). Total white blood cell (WBC) counts were measured using an electronic cell counter (Coulter model 2N, Coulter Electronics, Hialeah, FL). Differential WBC counts were made by counting 100 white cells from blood smears stained with Wright stain (Sigma Diagnostics, St. Louis, MO). Final WBC counts were corrected for nucleated red blood cells (nRBC), if present. Plasma cortisol concentrations were measured using a radioactive immunoassay kit (Kallestad Labs, Austin, TX). Plasma glucose and triglyceride concentrations were measured using a Reflotron clinical chemistry analyzer (Boehringer Mannheim Diagnostics, Indianapolis, IN).

Bacteriologic culturing was performed using 1-2 ml of blood obtained by cardiac puncture immediately after the animals were killed. Samples were incubated for 24-48 h at 37°C in brain-heart infusion broth. An aliquot was then transferred to blood agar

plates for an additional 24 h incubation. Pulmonary lavage fluid was obtained after death from rabbits inoculated via the intranasal route. Sterile saline (10 ml) was injected into the trachea and immediately withdrawn. An aliquot was then cultured of blood agar plates for 24 h.

Data were analyzed by using a two-way analysis of variance for repeated measures, with individual means compared using Fisher's least significant difference test for a priori comparisons (123). A significance level of $P < 0.05$ was accepted.

Experiment No. 4: Somnogenic actions of bacterial cell walls

Staphylococcus aureus SG511 from the culture collection of the Robert Koch Institute in Berlin (FRG) was grown in 2.5% Bacto-Peptone (Difco) and 0.5% NaCl medium, pH 7.2, in conical flasks aerated by vigorous agitation. When the culture reached an optical density of 0.5 at 578 nm, chloramphenicol (20 µg/ml) was added, and the incubation was continued overnight. Chloramphenicol treatment enhances O-acetylation (58) and the thickness (47) of staphylococcal cell walls. Bacteria were harvested, and cell walls were isolated as previously described (58). Briefly, the culture was centrifuged, and the cellular pellet was suspended in 2 M NaCl. The bacteria were then disrupted by agitation with glass beads. Membranes and noncovalently bound proteins were removed by treatment with 10 mg/ml sodium dodecyl sulfate (SDS) at 60°C for 30 min. Covalently bound proteins were removed by treatment with 0.02 mg trypsin in 0.1 M tris (hydroxymethyl) aminomethane (Tris)-HCl buffer, pH 7,

at 37°C for 24 h. After extensive washing (3x) in this buffer and three additional washes in water, the cell walls were lyophilized for storage.

To eliminate O-acetyl groups, lyophilized cell walls (maximum 20 mg) were suspended in 1 ml of 0.05 M NaOH for 1 h at 37°C, whereas preparations with intact O-acetyl groups were suspended in water under the same conditions. The cell walls were centrifuged at 11,600 xg for 10 min, and the supernatants were discarded. The pellets were washed three times with water. To prepare intravenous inoculations, cell walls were suspended in pyrogen-free physiological saline (PFS) (0.9% NaCl, Abbott Labs, North Chicago, IL) to provide the desired concentrations of cell walls in a total volume of 1 ml. The doses of staphylococcal cell wall material used (0.9 and 2.4 mg) contained 1×10^9 and 2.7×10^9 sacculi, respectively, as measured with a hemacytometer; this dose is similar to the active dose of heat-killed staphylococci previously used (7×10^9 colony forming units) (129).

Insoluble PG, purified from *Neisseria gonorrheae* strains FA19 and RD5 as previously described (112), was the source of extensively O-acetylated PG (O-PG) and non-O-acetylated PG (non-O-PG), respectively. Approximately 50% of the disaccharide peptide monomer subunits of strain FA19 PG are substituted with O-acetyl groups at C-6 of muramic acid, whereas strain RD5 PG completely lacks O-acetyl derivatives (90, 126). Sonicated (S) PG (S-O-PG and S-non-O-PG), prepared from O-PG and non-O-PG as described (106), were heterogenous mixtures of soluble fragments $>10^6$ D. To reduce trace levels of endotoxin, purified soluble PG preparations

in PFS were treated with conjugated polymyxin B agarose (Sigma Chemical, St. Louis, MO) for 30 min at 25°C; polymyxin B agarose conjugate was added in an amount equivalent to 1.1 mg of polymyxin B/ml. Polymyxin B-treated preparations were centrifuged at 500 g for 15 min to remove the insoluble polymyxin B conjugate.

In control experiments, the polymyxin B, as described above, removed ~24 µg/ml of authentic N. *gonorrhoeae* strain 1291 lipopolysaccharide (LPS, courtesy of M. Appicella, Buffalo, NY) from a solution that contained 25 µg LPS/ml before treatment, as assessed by Limulus amoebocyte lysate (LAL) tube assays (45). Final preparations of S-O-PG and S-non-O-PG subjected to this treatment contained >10 pg LPS/µg PG, as determined by LAL assay using gonococcal LPS as a standard. In addition, final PG preparations contained no detectable contamination by non-PG amino acids at a sensitivity that would have easily detected 0.1% (wt/wt). To prepare intravenous inoculations, 2.5 mg PG in 0.2 ml of water was added to 0.8 ml PFS to provide a final concentration of 0.5 mg PG/ml.

Test substances suspended in 1 ml PFS were injected into a marginal vein of the ear; in control recordings 1 ml PFS was administered. Colonic temperatures (T_{co}) and blood samples were obtained before IV injection and after the 6-h recording period. T_{co}s were measured using a flexible thermistor probe (Yellow Springs Instruments, Yellow Springs, OH) inserted 10 cm into the colon. Blood samples (1.0-1.5 ml) were collected from the central artery of the ear and were immediately transferred to VACUTAINER tubes containing EDTA (Becton Dickenson). Total white blood cells

(WBC) were counted with a cell counter (model 2N, Coulter Electronics, Hialeah, FL). For differential WBC counts, blood smears were stained with Wright stain, and 100 WBC were counted. WBC counts were corrected for nucleated erythrocytes, if present.

Control and experimental recordings were obtained on different days from the same rabbits; thus a within-subject experimental design was used. Effects occurring during the 6-h period after injection were evaluated with a nonparametric two-way analysis of variance (Friedman test). T_{co}, neutrophil, and lymphocyte numbers and cortisol concentrations were compared using the Wilcoxon matched-pairs signed-ranks test. An alpha level of $P < 0.05$ was accepted.

Experiment No. 5: Macrophage production of somnogenic muramyl peptides

Growth of bacteria. *Staphylococcoccus aureus* SG 511 of the culture collection of the Robert Koch-Institute was kept on 1.5% peptone agar dishes at 37°C. Cells from the dishes were used to inoculate a culture growing overnight at 37°C in 2.5% peptone (Bacto-Peptone; Difco Laboratories, Detroit, Mich.) and 0.5% sodium chloride in water. To monitor growth, the optical absorbance at 578 nm (A) of liquid cultures was measured. The precultures were used to inoculate a second culture at a starting A=0.05. At A=0.125 cell walls were labelled with 200 ml of the cell wall specific (58) N-acetyl-D-(1-^{14}C)-glucosamine (1.924 GBq/mmol, 9.25 MBq/1.25 ml, Amersham-Buchler, Braunschweig, FRG). At A=1.0, bacteria were harvested by centrifugation at 4°C for 10

min at 3000 xg, resuspended in 150 mM NaCl to a concentration of 10^8 cells/ml and used in phagocytosis experiments as described below.

Macrophage processing of S. aureus. Bone marrow cells were removed from femur and tibia of 2 month old (30g) female Balb/C mice and cultivated at 37°C and 5% CO_2 in Dulbecco modified minimal essential medium containing 5% fetal calf serum, 15% horse serum, and 20% L cell supernatant as colony stimulating factor in Petriperm dishes. Each day 1 ml of fresh medium was added. By day four, macrophages were adherent while other cells were in the supernatant. On day seven or eight, the medium was completely exchanged thereby removing non-adherent cells. On day ten, macrophages were used for phagocytosis experiments. *S. aureus* were opsonized with 25% specific rabbit antiserum for 20 min at room temperature immediately before being added to the macrophages. The opsonized bacteria were added to the adherent macrophages at a concentration of 100 bacteria per macrophage. After 30 min, the macrophages were washed three times with 1 ml Dulbecco medium to remove non-ingested bacteria from the macrophages. The cells were then cultivated in Dulbecco medium with serum as above (containing 100 mg/ml Streptomycin and 100 U penicillin/ml). After 3, 24, 48, or 96 hours, the medium was withdrawn, pooled, frozen at -20°C and lyophilized.

Cell free degradation of staphylococcal cell walls. Cell walls were isolated from staphylococci that were grown overnight under the influence of 20 µg/ml chloramphenicol. Chloramphenicol and radioactive marker as above were added at

A=0.5. Bacteria were harvested as above, suspended in 2 M NaCl and vigorously agitated for 5 min in a glass bead filled cell mill. The completeness of bacterial disintegration was verified by inspection under microscope after Gram staining. The cell walls were separated from the glass beads with a sintered glass filter and stirred in 1% sodium dodecyl sulfate for 30 min at 60°C to remove membranes and noncovalently bound proteins. After washing six times in H_2O (centrifugation at 10000 xg for 10 min) the cell walls were treated with 0.02% trypsin in 0.1 M Tris/HCl pH 7 buffer for 24 hours at 37°C to remove covalently bound proteins. After washing three times in buffer and three times in H_2O as above, the walls were suspended in water and lyophylized.

To render staphylococcal cells walls susceptible to degradation by lysozyme, O-acetyl groups were removed from the cell walls by 1 hour treatment with 0.05 N NaOH at 37°C(58) and three times washing in water as above. Cell walls (5-20 mg) were suspended in 10 ml 150 mM, pH 5 sodium acetate buffer containing 1 mg/ml hen egg-white lysozyme and shaken for 24 hours at 37°C.

Amino acid and amino sugar analysis. Lyophilized macrophages supernatants and isolated cell walls (prepare as described above) were hydrolysed in 4 N HCl at 105°C for 16 h in sealed tubes. After drying under a stream of nitrogen, the hydrolysate was subjected to amino acid and amino sugar analysis on a Biorad LC 6000 amino acid analyzer. The buffer program was adjusted such that muramic acid could be detected. An equal aliquot of the sample was used to determine the radioactivity in that sample.

Sample purification. The lyophylized macrophage supernatants (0.2-0.25 g) were dissolved in 1 ml of water and centrifuged at 1000 xg for 10 min to remove precipitated salts and proteins. Pellets were resuspended in 1 ml water and centrifuged again. The supernatants were combined. These supernatants and the lysozyme reaction solutions were then treated in the same way except as noted. To remove proteins by acid-acetone precipitation, a 15-fold volume of 1 N HCl:acetone (3:100) was added to the supernatants of the preceding step and to the lysozyme reaction solution. After 1 h at 4°C, precipitated proteins were sedimented by centrifugation at 1000 xg for 10 min. The precipitates were washed in the same volume of 0.1 N HCl:acetone (20:100) and, after 1 h at 4°C, centrifuged as above. The combined supernatants of the centrifugation steps were dried in a rotary evaporator. After dissolution in 1 ml water, hydrophobic components were removed by passage through a C_{18} solid phase extraction cartridge.

Samples were dissolved in 2-5 ml 0.05 N acetic acid, introduced to gel filtration chromatography on a 90 x 1.5 cm Fractogel TSK HA40[F] column (MW range 100 - 10000 for peptides, Alltech), and eluted with 2 ml/min 0.05 N acetic acid. Fractions (1.5 or 2.0 ml) were collected and aliquots of these were used to determine their radioactivity. Fractions of low molecular weight were pooled as indicated (Figure 18, Table 11), dried, and tested for biological activity.

Low molecular weight products obtained by gel filtration from macrophage supernatants were further separated by reversed phase

chromatography. Aliquots of the low molecular weight fractions (20 - 38, Figure 18) obtained with gel filtration were pooled, dried, and suspended in 0.5 ml 0.05% trifluoroacetic acid (TFA) in H_2O. Aliquots of 0.1 ml were introduced to a 25 cm x 4.6 mm Rainin Dynamax Microsorb C_{18} column with precolumn (type of precolumn) in a Varian LC 5000 instrument. Solvent A was 0.05% TFA in H_2O, solvent B 0.035% TFA in acetonitrile. The column temperature was 25°C. The concentration of solvent B was increased linearly from 0% at time 0 to 25% at 30 min, and then to 80% at time 35 min where it was held for 5 min. Fractions of 0.5 ml were collected and aliquots were used for determination of radioactivity. Fractions containing radioactivity were pooled as indicated (Table 12), dried, and tested for biological activity.

Test samples were dissolved and diluted to appropriate concentrations in aCSF in nonpyrogenic sterile saline (Abbott) and heated to 60°C for 30 min to destroy possible residual contaminant activity of heat labile macrophage products such as cytokines. Samples (25 µl) were then injected ICV in rabbits over a 1 min period between 0800 and 1000h.

RESULTS

Experiment No. 1

IL1α dose-dependently enhanced NREMS (Table 2, Fig. 1); Friedman's test across doses was significant for W and NREMS. After about a 1-h delay, increases in NREMS induced by IL1α were

manifest, then they persisted for about 5-6 h, depending on the dose; Friedman's test across 6 h was significant for NREMS for each dose. IL1α also significantly enhanced averaged EEG-delta wave voltages after all three doses (Fig. 2). EEG-delta wave values are influenced by both the amount of time spent in NREMS and the amplitude of individual delta waves. They also can serve as an objective method to validate NREMS values determined by visual scoring (Fig. 1). An alternative method of analysis determines EEG-delta voltage only during NREMS episodes (Table 3); these values are indicative of the maximal EEG amplitudes and do not include voltages during W, REMS or transitions between states. Higher maximal delta wave amplitudes are thought to reflect a greater intensity of NREMS since supranormal EEG slow waves are present after sleep deprivation (104). IL1α dose-dependently enhanced EEG-delta wave voltage during NREMS (Friedman's test across doses). IL1α also enhanced EEG theta wave voltages after the highest dose; voltages in alpha and beta EEG frequency bands and in the theta band after lower IL1α doses were not affected.

In contrast to the IL1α-enhanced NREMS, IL1α suppressed REMS. REMS reductions were evident in the first hour postinjection and persisted throughout the assay period (Fig. 1); these effects were also dose-dependent (Friedman's test across doses). Although the behavior of the recipient animals was not systematically investigated in this study, no abnormal behaviors were observed. Animals continued to cycle through the various stages of vigilance in a normal manner and, when handled at the end of the test

period, appeared normal. Similar to nontreated animals, they could easily be aroused from sleep when disturbed.

The IL1α used in this study also induced dose-dependent fevers (Friedman's test across doses) (Fig. 3). All three doses used induced a rapidly developing fever that peaked at about 1.5°C above normal values 1 h after injection. After the highest dose used, T_{br} continued to increase for about 3 h, plateauing at about 2.7°C, 3 h after the injection. Six hours after injection, both T_{br} (Fig. 3) and T_{co} (Table 2) remained elevated after all three doses of IL1α. If the IL1α was heat treated before injection, its pyrogenic (Fig. 3) and somnogenic effects (Table 2) and its effects on EEG slow waves (Fig. 2) were abolished.

For comparative purposes, the effects of two doses of IL1β are included in Table 2. IL1β induces enhanced NREMS and T_{br} and reduces REMS; these data are similar to those previously reported (73, 121). IL1β also enhanced EEG slow-wave amplitudes (data not shown).

Several synthetic fragments of both IL1α and IL1β were also tested for somnogenic and pyrogenic activity. The IL1α fragments $IL1\alpha_{115-148}$ and $IL1\alpha_{223-250}$ failed to alter sleep or T_{br}. However, these fragments possessed other biological activities. Peptide 115-148 stimulated T cell proliferation, and peptide 223-250 stimulated PGE_2 production by ARSC (Table 4). Similarly, IL1β peptides 117-134, 178-207, 199-228, and 247-269 also failed to alter sleep or T_{br}; some of the fragments possessed other biological activities. Peptides 178-207, 199-228, and 247-269 stimulated T cell proliferation (Table 4).

In contrast to the results obtained with other fragments, $IL1\beta_{208-240}$ enhanced NREMS, EEG slow-wave amplitudes (not shown), and T_{br} after ICV injection of 25 μg (Table 2). The time courses of these effects were similar to those observed after injection of hu-rIL1β (73, 121). This dose of $IL1\beta_{208-240}$ also reduced REMS, but these effects were not significant. As described above for IL1α, $IL1\beta_{208-240}$ also did not appear to induce abnormal behavior, although this was not systematically documented in this study. $IL1\beta_{208-240}$, although not mitogenic for T cells, did stimulate PGE_2 production by ARSC (Table 4).

Experiment No. 2

GH secretion from perifused adult male rat pituitary cells was significantly ($p < 0.01$) stimulated by the HPLC-purified 3'-5' CP at each concentration tested. The first exposure to the 3'-5' CP (0.1 nM), resulted in the largest GH release (approximately 223% over the basal secretion rate). The response was rapid in onset, and lasted about 20 minutes, after which the secretion rate returned to the prestimulation baseline. Two hours later, a second stimulation of the preparation, or a third stimulation three hours later with larger doses of the 3'-5' CP also elicited GH release, though the response was not as large as that initially observed (Fig. 5).

Icv administration of 1 nmol/kg 3'-5' CP promoted sleep. Increases in NREMS were significant for the 6-h recording period; REMS increased in postinjection hour 2 (Fig. 6, Table 5). The

purified 3'-5' CP obtained after HPLC purification had sleep effects similar to the original sample (Table 5).

The hydropathic pattern of GHRH (14-25) is opposite to that of the 3'-5' CP, as predicted (Fig. 7a). However, by shifting the sequence of the 3'-5' CP by two amino acids to the C-terminal along the GHRH molecule, the hydropathic patterns of the two peptides match in the C-terminal heptapeptide region, corresponding to rGHRH (21-27) (Fig. 7b). According to Chou & Fasman (25) analysis of the 3'-5' CP, the C-terminal 6 amino acids may initiate an alpha-helix (P_a=1.168 > P_b=1.12). This helix eventually involves the C-terminal 7-8 amino acids of the 3'-5' CP, the ones with matching hydropathic pattern with rGHRH (21-27). The analysis predicts alpha-helical structure also for rGMRH (21-27) (P_a=1.245 > P_b=1.060).

Experiment No. 3

Somnogenic effects. The somnogenic effects of inoculation with 0.7 ml of sterile pyrogen-free saline were similar after iv, im, in, and sc administration (n = 2 for each route), and data were therefore pooled for group analysis (Fig. 8). Saline administration did not significantly alter time spent in SWS or delta wave amplitudes (DWA) during SWS. REMS was sporadically reduced in saline-treated rabbits, although a clear circadian pattern of REMS still occurred.

In contrast to the lack of effect of saline administration, inoculation with viable *P. multocida* induced biphasic changes in SWS after all four routes of administration (Fig. 9). SWS was

markedly enhanced during hours 0-4 after challenge by iv, im, and sc routes and then decreased relative to the comparable control period. In contrast to these effects, challenge by the intranasal route enhanced SWS during hours 4-10 after inoculation and suppressed sleep during hours 20-40.

Effects on DWA during SWS after inoculation with *P. multocida* temporally paralleled the effects on SWS in that DWA was sequentially enhanced and suppressed after iv and im challenge (Fig. 10). A significant increase in DWA did not develop in rabbits challenged intranasally with *P. multocida,* although these animals did demonstrate reduced DWA during SWS for hours 12-48 after inoculation. In contrast, rabbits inoculated by the sc route developed large increases in DWA for up to 6 h after challenge, but the subsequent decrease in DWA was somewhat reduced.

Inoculation by all four routes induced an inhibition of REMS that persisted throughout most of the postinoculation period (Fig. 11). However, the latency with which this inhibition developed varied with the route of administration. Thus REMS attenuation developed during the first 2 h after inoculation via the iv, im, and sc routes but during the 2- 4-h period after intranasal challenge.

Pyrogenic and hematologic effects. The hematologic effects of inoculation with 0.5-1.0 ml of sterile pyrogen-free saline were similar after iv, im, in, and sc administration (n =2 for each route), and data were therefore pooled for group analysis (Table 6). Saline administration did not significantly alter

rectal temperature, total number of WBC, number of circulating nRBC, or plasma concentrations of cortisol, triglycerides, or glucose. However, saline administration did induce mild neutrophilia 6 h after inoculation.

In contrast to the modest effects of saline administration, iv inoculation of rabbits (n = 8) with viable *P. multocida* induced dramatic changes in most of the parameters that were measured (Table 7). Infected rabbits developed a 1-1.5°C increases in rectal temperature that persisted for up to 24 h after inoculation. Total WBC counts demonstrated an initial decline, followed by a return to normal numbers. Differential analysis indicated that neutrophilia, lymphopenia, and increased numbers of circulating nRBC were present during hours 6-24 after inoculation. Plasma cortisol concentrations were also significantly elevated during this time. Plasma triglyceride concentrations had increased substantially by 24 h after inoculation, and transient hypoglycemia was observed.

Rabbits challenged via the im, in, or sc routes also developed fever and hematologic changes. Fever persisted throughout the 48-h period after inoculation but was biphasic after sc challenge (Fig. 12).

Significant hematologic alterations were observed in rabbits challenged with *P. multocida* (Fig. 13). A decrease in total WBC numbers that persisted for up to 48 h after inoculation was induced by im administration. WBC numbers also decreased after sc challenge, although this effect, like the fever, was biphasic. Total WBC counts were not significantly altered after in

challenge. Numbers of nRBC were not significantly increased after sc challenge, increased dramatically within 6 h after im challenge, and exhibited large increases with a latency of 36-48 h after in inoculation. In addition to these effects, the distribution of WBC was dramatically altered after challenge with *P. multocida* (Fig. 14). All three inoculation regimens reversed the proportionate numbers of neutrophils and lymphocytes in the peripheral blood, resulting in neutrophilia and lymphopenia. This effect occurred with a slightly longer latency after intranasal challenge but demonstrated a shorter duration after challenge by the other two routes.

Inoculation of rabbits with *P. multocida* also stimulated alterations in other physiological parameters that were evaluated (Fig. 15). Challenge by any of the three routes increased plasma cortisol concentrations, although the increase was transient after sc challenge. A marked increase in plasma triglyceride concentrations that was similar to that observed after iv challenge was induced by im inoculation. In contrast, inoculation via the intranasal route elicited a relatively smaller increase after a 36-h delay, and concentrations were not significantly increased after sc challenge. Hyperglycemia occurred 48 h after challenge by any route.

Blood samples obtained by cardiac puncture after the animals were killed were cultured for bacterial growth (Table 8). All rabbits killed 24 h after challenge via the iv route were septicemic at the time of death. However, samples obtained from animals inoculated via other routes and killed 48 h after

challenge were predominantly negative. Pulmonary lavage fluid collected 48 h after inoculation from rabbits challenged intranasally was found to contain *P. multocida* in most animals tested.

Experiment No. 4

Intravenous injection of rabbits with *S. aureus* cell walls (0.9 or 2.4 mg/rabbit) increased the amount of time spent in SWS and the amplitude of EEG delta waves, while the amount of time spent in REMS was reduced during the 6-h period after injection (Table 9). Pronounced neutrophilia and lymphopenia (Table 10) were also induced by these cell wall preparations. Injection of de-O-acetylated *S. aureus* cell wall significantly enhanced SWS and EEG delta-wave amplitudes only at the higher dosage (2.4 mg) (Table 9). However, both doses of this preparation reduced REMS, enhanced T_{co} (Table 9), and induced lymphopenia and neutrophilia (Table 10).

The time courses of responses elicited by acetylated and deacetylated cell wall preparations were very similar. The effects on SWS (Fig. 16 A), REMS (Fig. 16 B), EEG delta-wave amplitudes (Fig. 16 C), and T_{brs} (Fig. 16 D) developed 2-3 h after injection and then remained above base-line values for the remainder of the 6-h recording period.

N. gonorrhoeae PF preparations enhanced SWS (Table 9, Fig. 17 A) and amplitudes of EEG delta-waves (Table 9, Fig. 17 C) and reduced REMS (Table 9, Fig. 17 B). The sleep effects were apparent by the second postinjection hour and persisted for 2-3 h.

SWS and EEG delta-wave amplitudes returned to control values by 5-6 h after injection (Fig. 17, A and C), while the reduction in REMS persisted throughout the experiment (Fig. 17 B). T_{co}s were not elevated 6 h after injection (Table 9). However, biphasic fevers did occur during hours 1-4 postinjection, as determined from T_{br}s (Fig. 17 D). The magnitudes of sleep and temperature effects were considerably larger than those induced by staphylococcal cell walls (compare Fig. 16 with Fig. 17). Lymphopenia and neutrophilia also occurred after injection with soluble PGs, and these changes were similar to those elicited by *S. aureus* cell walls (Table 10).

Experiment No. 5

Sample preparation. Aliquots of unprocessed macrophage supernatant or of isolated cell walls were used to determine the amounts of radioactive label and of muramic acid in the preparations using amino acid analysis and measurement of radioactivity. The amount of ^{14}C-radioactivity per nmol Mur found in the macrophage supernatants was 807 dpm; 208 dpm of ^{14}C-radioactivity per nmol of Mur was found in the isolated cell walls. These ratios were used to calculate doses for the biological tests. It is noted that this ratio is not necessarily the same in all fractions after chromatographic separation. Further, due to the high specific biological activity of some of the samples tested, the amount of radioactivity was to low to allow accurate measurements. Nevertheless, virtually 100% of the radioactive marker in the samples applied were recovered from the

initial cleaning steps, including centrifugation of the macrophage supernatants, acid-acetone extraction and solid phase extraction of macrophage and lysoszyme digests (data not shown).

After acid-acetone extraction and C_{18} solid phase extraction, large molecular weight components from samples of macrophage digests of viable staphylococci and of lysozyme digests of isolated staphylococcal cell walls were removed using gel filtration. The gel filtration fractions from both samples contained radioactivity in two major areas (Fig. 18); one near the exclusion volume (high molecular weight) and the other near the permeation volume of the column (lower molecular weight). The proportion of radioactivity found in the low MW fractions after macrophage digestion was greater than that observed after lysozyme digestion (Fig. 18). Only the pooled low molecular weight components marked by filled columns in Fig. 18 were used in biological tests and, in the case of the macrophage digestion, for further purification.

Macrophage digestion products of low molecular weight obtained by gel filtration were further separated on reversed-phased HPLC. All radioactivity applied to the column eluted within 40 min (Fig. 19). The majority of radioactive components eluted early during the gradient run, as expected from the relatively hydrophilic chemical composition of bacterial cell wall components. Fractions were pooled as indicated in Table 12 and tested for biological activity.

Biological effects. The ICV injection of an aliquot containing radioactivity corresponding to an amount of 350 pmol

muramic acid from pooled fractions 20-38 of the gel filtration step of the macrophage supernatant induced significant increases in the time spent in SWS and a reduction of REMS over the six hour recording period, and an elevated T_{co} of 1.8° C at the end of the recording period (Table 11, sample #1). The sleep effects were not apparent in the first postinjection hour; they became manifest during the second postinjection hour and were still evident at the end of the recording period (Fig. 20). Also, the amplitudes of EEG δ-waves were increased from hour two postinjection until the end of the recording period (Fig. 20 C). The time courses of febrile responses were similar; they started during the second hour postinjection then persisted for the remainder of the recording period.

In a control experiment, samples were produced by a cell free system consisting of lysozyme and isolated bacterial cell walls and were separated by gel filtration. Chromatographic fractions were pooled into two lots (Fig. 18, Table 11, samples 2 and 3) and injected ICV into rabbits. The injection of both these samples significantly enhanced SWS, suppressed REMS, and increased T_{co} (Table 11, samples 2 and 3). The time course of the sleep effects were similar to those observed with macrophage supernatants in that there was a delay of about one hour before effects were observed and the effects were still evident at the end of the six hour recording period.

The majority of the HPLC fractions tested were not associated with significant sleep responses at the doses tested (Table 12). Only a single sample (pooled fractions 24-26, Table 12)

significantly enhanced SWS and reduced REMS, at a dose of 10 pmol. After injection of this sample, excess SWS, enhanced δ-wave amplitudes and reduced REMS were first observed in the third hour postinjection and then persisted for the rest of the assay period (Fig. 21). This sample was also pyrogenic at both tested doses (Table 12). The rise in T_{br} started in the second postinjection hour and continued throughout the recording period (Fig. 21 D).

An other sample (pooled fractions 43-47, Table 12) caused increased SWS and δ-wave amplitudes and was pyrogenic at a dose of 50 pmol. However, it had no effect on REMS and 10 pmol of this sample was inactive in regard to sleep and caused only a moderate increase in T_{co} (Table 12). Pooled fractions 50 and 51 induced increased δ-wave amplitudes but this was not accompanied by changes of sleep stages (Table 12). Several other fractions contained pyrogenic activity (Table 12). These fractions, interestingly, eluted more towards the hydrophobic end of the elution profile.

In all cases where changes in sleep were observed, the nature of the sleep resembled that of normal physiological sleep, in that sleep remained episodic, sleep stages were accompanied by transient changes in T_{br}, and the behavior of the animals, although not systematically evaluated, seemed normal.

DISCUSSION OF SPECIFIC EXPERIMENTS

Experiment No. 1

The two forms of IL1, IL1α and IL1β have been identified in various species. Generally, IL1β is the prevalent form in both the tissue fluids and the plasma (31). In cell cultures and tissues, distributions of IL1β and IL1α mRNA are often similar, but sometimes IL1β or IL1α mRNA seems to be more abundant than the other (28, 127). Although only distant homologies were found between the amino acid sequences of the two forms of IL1, both of them evidently bind to the same receptors (35). Receptor affinity of IL1α may be less than that of IL1β, however, this may also vary with the tissues (24, 32). The common receptor may explain the similarities in the spectrum of biological activities of the two forms of IL1. In most assays, IL1α and IL1β are equipotent (115) or IL1α is only slightly less potent than IL1β (24, 32). In a few *in vitro* tests, however, substantial differences were found between the activities of IL1β and IL1α (3, 116). *In vivo*, only IL1β promotes production of factor VII (22), and vascular permeability is increased by IL1β but is hardly affected by IL1α (91). In contrast, IL1α is more active than IL1β in stimulating resorption of rat long bones (85). Finally, the two forms of IL1 clearly differ in a central nervous system action; through the stimulation of hypothalamic corticotropin releasing factor release (4, 117) IL1β elicits adrenocorticotropic hormone secretion whereas IL-1α has no similar effects (132). It was of interest,

therefore, to show that the promotion of sleep, a prominent central action of IL1β, is also shared by IL1α.

Increases in NREMS, suppression of REMS, and fever in response to ICV administration of IL1α were similar to those observed after IL1β in this study and in previous experiments (73, 121). IL1α, however, was active at a dose as low as 0.01ng indicating that the somnogenic potency might be greater than that of IL1β, though such comparisons are difficult since the fraction of sample protein that is active IL1α or IL1β is unknown.

Enhancement of sleep elicited by IL1 is regarded as one of the systemic symptoms, collectively termed the acute phase response, which occur after infectious or tissue injuries. Inoculation of gram negative or gram positive bacteria induces enhanced NREMS similar to that found after administration of IL1 (130). The effect is attributed, in part, to substances released from bacterial cell walls; e.g., muramyl peptides and endotoxin. Intravenous injection of bacterial cell walls, and ICV or systemic administration of muramyl peptides or endotoxin elicits fever, promotes NREMS and suppresses REMS as found after IL1 (reviewed, 71). Both muramyl peptides and endotoxin stimulate IL1 secretion, and IL1 is postulated to mediate the somnogenic and pyrogenic effects of these substances. It seems, however, that the forms of IL1 produced varies with the stimuli. Heat-killed *Staphylococcus epidermidis*, a gram positive bacterium, induces more IL1β than IL1α whereas, endotoxin elicits more IL1α than IL1β production (38). Since both forms of IL1 are somnogenic, it is possible that enhancements in NREMS after muramyl peptides (infections with gram

positive bacteria) and after endotoxin (gram negative bacteria) are mediated primarily through IL1β and IL1α, respectively.

Relatively small (about 4 kd) peptides with IL1-like activities were purified from the plasma of febrile patients (33) and from the urine of healthy humans (67). It was suggested that the peptides might represent short cleavage products of the mature IL1. These observations initiated experiments aiming to elucidate structure-activity relationships for IL1 and, eventually, to produce active IL1-like peptides. It seems that both the amino terminus and the carboxy terminus are important for binding of the 17 kD IL1 to IL1-receptors (98, 140), and antibodies recognizing these portions render IL1 inactive (94). The thymocyte proliferation assay may correlate with the immunomodulatory action whereas stimulation of PGE_2 release by human synovial cells is regarded as an indicator for the inflammatory effects of IL1 (30). These activities of IL1 are not necessarily associated in the IL1 fragments. For example, an IL1β fragment, the peptide 163-171, is active in thymocyte proliferation test (1), and though it increases hyaluronic acid release in fibroblasts (108), the peptide fails to stimulate PGE_2 production (1, 108). It was concluded that the different sites of the IL1 molecule might be responsible for distinct biological activities, alternatively, the molecule has only one active site, but the inflammatory effect also requires the integrity of other parts of the molecule (1).

Although the doses of the peptides used in the thymocyte proliferation test and the PGE_2 production test were not always comparable and not all of the peptides were tested in both assays,

the immunostimulatory and inflammatory actions were clearly separated for a few peptides in our experiments. Thus, the IL1α peptide 115-148 and the IL1β peptide 178-207 were effective only in the thymocyte proliferation assay. In contrast, the IL1α peptide 223-250 and the IL1β peptide 208-240 increased PGE_2 production by fibroblasts without promoting proliferation of thymocytes. These findings suggest that immunostimulation and inflammatory activities are, in fact, associated with different regions of the IL1 molecule.

As indicated by the doses required for activity, the IL1 fragments were far less potent than the mature IL1 both *in vitro* and *in vivo*. The amounts of peptides available did not allow ICV testing doses higher than 10-25 μg. It is possible, therefore, that some of the peptides might have sleep or body temperature effects when administered in very large doses. Out of the seven peptides, there was only one, the IL1β peptide 208-240, which promoted NREMS and elicited fever in rabbits. It was recently reported that antibodies recognizing the region 218-243 of IL1β moderately inhibit IL1β actions *in vitro* (94). This finding indicates that this region might, in fact, be involved in the biological actions of IL1β.

IL1β peptide 208-240 stimulated PGE_2 production by fibroblasts, enhanced NREMS, and elicited fever, but did not stimulate proliferation of thymocytes. It is possible, therefore, that the pyrogenic and somnogenic effects are independent from the immunomodulatory actions of IL1. The intriguing question is whether the sleep effects and fever are linked to the increased

synthesis of PGE_2. Several lines of evidence indicate that PGE_2 is involved in the mediation of fever. PGE_2 is known to elicit fever (reviewed, 26). IL1 stimulates eicosanoid, including PGE_2, production by astrocytes (52), and PGE_2 levels in the CSF increase after administration of pyrogens (5, 27). It has been suggested that IL1 acts on the organum vasulosum laminae terminalis (12) by releasing PGE_2 which elicits fever (34). Therefore, the *in vitro* PGE_2 stimulatory activity of the IL1β peptide 208-240 is in a good agreement with the fever obtained *in vivo*. However, the IL1α peptide 223-250 also stimulated PGE_2 production by fibroblasts and yet it was ineffective when injected ICV. The discrepancy might be explained by the observation that IL1-stimulation of PGE_2 production *in vitro* is not always coupled by similar effects *in vivo* (54).

The importance of the stimulation of PGE_2 production for the sleep effects is not clear. Increases in NREMS and fever in response to IL1 can be separated by means of administration of antipyretics (73), therefore, the mechanisms of fever are not likely to be involved in the promotion of sleep. Finally, instead of enhancing sleep, administration of PGE_2 seem to increase wakefulness, though in rats a sleep promoting capacity has been attributed to PGD_2 (53). *In vitro* IL1 stimulates PGD_2 production by astrocytes (141). Regardless of whether IL1 and/or an IL1 fragment alter sleep via PGs, it is currently clear that both IL1α and IL1β and one fragment of IL1β (208-240) have the capacity to enhance NREMS.

Experiment No. 2

The present results indicate that the 3'-5' CP of rGHRH is biologically active both *in vitro* and *in vivo*. Human and rat GHRH are well established stimulators of GH secretion (e.g. 15, 50, 111, 124). The GH releasing effect of human GHRH, which shares 74% sequence homology with rGHRH in the N-terminal 27 amino acids, is not strictly species specific; hGHRH stimulates GH secretion from rat pituitary cells *in vitro* (2). Human GHRH (1-40) is also a potent promoter of NREMS in rats and in rabbits (36, 102). This effect is not mediated through GH release, instead it is attributed to actions of GHRH on basal forebrain somnogenic structures (102).

The present study shows that the 3'-5' CP shares these two biological activities with GHRH: stimulation of GH release and stimulation of sleep. There is no experimental evidence that the 3'-5' CP acts on the GHRH receptor. However, the GHRH-like biological activity of the 3'-5' CP in two different species, two different effects, the direct effect on pituitary cells, and that the sequence was derived from the mRNA of GHRH, are consistent with the hypothesis that this peptide might act via the GHRH receptor. In view of the CP theory, according to which the 3'-5' CP should resemble the binding site of the GHRH receptor, the GHRH-like effects of the 3'-5' CP were unexpected. However, based on Chou and Fasmen analyses (Fig. 7), both the 3'-5' CP (5-12) and the rGHRH (21-27) form alpha-helices with similar hydropathic patterns. Furthermore, the hydrophilic and hydrophobic amino acids are arranged in such a sequence that these peptide regions

form amphiphilic alpha-helices, the hydrophilic amino acids occupying one face of the helix, while the hydrophobic amino acids face the other side of the molecule. The amphiphilic secondary structure of peptide hormones is strongly correlated with their biological activities. Thus, model peptides, designed to minimize sequence homology, while optimizing the amphiphilicity of the secondary structure, may have higher biological activity than the naturally occurring analog (64). The 3'-5' CP may, therefore, be an analog of the amphiphilic secondary structure of rGHRH (21-27). As, according to the CP theory, the 3'-5' CP is supposed to bind rGHRH as a GHRH receptor, but it also appears to bind to the receptor being homologous with a predicted active site of GHRH, one may hypothesize that GHRH receptors may aggregate through 3'-5' CP-like "patches," and this aggregation could be regulated by GHRH.

In conclusion, the present findings suggest that the CP theory may be a useful tool in designing biologically active peptides. However, based on the present studies, it is not clear whether the biological activity of the 3'-5' CP is related to its CP nature, or to being an analog of the amphiphilic secondary structure of rGHRH (21-27), or exerts its effects on a different receptor. Further studies are required to elucidate these possibilities.

Experiment No. 3

These experiments demonstrate that rabbits challenged with a natural pathogen administered via normal routes of infection

exhibit marked alterations in sleep patterns. Inoculation of rabbits with the Gram-negative bacterium *P. multocida*, the cause of a variety of disease syndromes in rabbits (44), elicits biphasic enhancement and suppression of SWS and an attenuation of REMS. These effects are similar to those previously described after challenge with other microbial organisms administered via the iv route (129-130). In addition, the temporal pattern of a rapid onset and short duration of SWS enhancement that developed after iv, im, and sc inoculation with *P. multocida* is similar to the pattern observed after iv challenge with another Gram-negative bacterium, *Escherichia coli* (130). However, the specific patterns of sleep alterations that occurred after challenge with *P. multocida* varied with the route of inoculation. Challenge by iv and im routes induced robust effects that occurred with short latency and long duration. In contrast, the effects of intranasal administration were temporally delayed relative to the effects of challenge by other routes. Challenge by the sc route was associated with an initial enhancement of SWS and DWA, but the subsequent depression of these parameters that accompanied challenge by other routes was less marked.

Inoculation of rabbits with *P. multocida* also induced fever, leukopenia, neutrophilia, lymphopenia, increased numbers of circulating nRBC, and increased plasma concentrations of cortisol, triglycerides, and glucose.

These effects were qualitatively similar after challenge by any route, although the magnitude and temporal pattern varied with the route of administration. Inoculation by iv and im routes

rapidly induced large changes in most of the parameters evaluated. After sc challenge, these effects occurred with a similar latency, but the magnitude and duration were reduced. In contrast, the effects of intranasal administration were similar in magnitude to the effects of iv or im challenge, but were temporally delayed relative to the effects of challenge by other routes.

The somnogenic and hematologic sequelae of infectious challenge in rabbits thus, appear to be roughly correlated. These challenge regimens that induced a severe infectious condition, as demonstrated by robust clinical alterations that occurred with short latency and long duration, were accompanied by marked alterations in normal sleep patterns, whereas regimens inducing more transient clinical signs were accompanied initially by enhanced SWS, but the subsequent attenuation of SWS was less robust. The smaller magnitude of the effects that resulted from sc challenge could represent either clearance of organisms from the tissue spaces or containment of organisms at the site of inoculation. In contrast to the rapid physiological response to iv, im, and sc challenge, a longer latency to induce enhanced somnolence and some hematologic effects was observed after intranasal challenge. Moreover, the enhanced somnolence was not accompanied by increased DWA. This time course could reflect a delayed or progressively developing host immune response to organisms in the air spaces relative to a more rapid response elicited by organisms in the circulation or tissue spaces.

Several observations suggest that the somnogenic effects of bacterial challenge are mediated in part by components of the

bacterial cell wall. Intravenous injection of rabbits with isolated bacterial cell walls (61) or with purified lioppolysaccharide (endotoxin) from Gram-negative bacterial cell walls (76) also enhanced SWS and reduced REMS for 3-6 h after injection. In more chronic studies, iv inoculation of rabbits with heat-killed *E. coli* induced sequentially enhanced and reduced sleep in a temporal pattern identical to that induced by inoculation with the viable organism (130). Furthermore, persistent septicemia does not appear to be essential to the maintenance of either the somnogenic or hematologic changes induced by bacterial challenge, given the observation that only a low proportion of animals were septicemic 48 h after challenge when sleep alterations, fever, and hematologic abnormalities were still ongoing.

The hematologic effects observed in rabbits challenged with *P. multocida* are well-known indexes of acute infectious disease. Increased plasma cortisol concentrations commonly accompany septicemia and other forms of stress (97, 110) and could, in part, mediate the changes in WBC distribution that were observed (56). However, increased cortisol concentrations did not develop after inoculation with saline and thus, do not reflect nonspecific stress of the experimental paradigm. The increased circulating triglyceride concentrations that develop in rabbits challenged with *P. multocida* probably did not reflect an overall metabolic imbalance in that significant changes in plasma glucose concentrations did not closely parallel the triglyceride alterations. Hypertriglyceridemia has previously been described

in rabbits challenged with various infectious agents (42, 46, 128) and may be mediated via the inhibition of lipoprotein lipase by tumor necrosis factor (TNF) (6, 114). Interestingly, TNF is also known to be somnogenic in rabbits (121).

In summary, these experiments demonstrate that rabbits challenged with a natural pathogen administered via normal portal of entry exhibit somnogenic, pyrogenic, and hematologic changes similar to those previously described in rabbits challenged intravenously with other microbial organisms (129-131). The magnitude and temporal pattern of these effects varies with the route of administration. Moreover, those routes of administration that result in more severe physiological manifestations of clinical disease also seem to elicit more robust effects on sleep.

Experiment No. 4

The PG preparations used in this study increased the time spent in SWS, EEG delta-wave amplitudes, and T_{co} and reduced the time spent in REMS. The effects of *S. aureus* and *S. aureus* PG injection on leukocytic responses are well established (125, 129, 138), and the results presented here are in agreement with those previously reported. Intravenous injection with streptococcal mucopeptide has previously been demonstrated to inhibit REMS and to induce fever (92-93) in rats and rabbits. Although the effects of bacterial infection (129, 130) and muramyl peptides (74-75) on SWS were unknown at that time, it was concluded that the streptococcal cell wall was responsible for fever induction. The presented data are in agreement with that conclusion and extend it

by demonstrating that PG is also responsible for the enhancement of SWS that is so prominent during infection or after muramyl peptide treatment.

Relative to the effects observed after injection of *S. aureus* cell walls, both gonococcal PG types used induced large but short-lasting effects on sleep and body temperature. These differences could be due to differences in the cell wall structures of the two organisms. Lower doses (0.5 mg/rabbit) of *N. gonorrhoeae* PG were injected because these preparations do not contain teichoic acid. In addition, in contrast to the thick, insoluble staphylococcal cell wall, gonococcal PG was already in soluble form when administered. The soluble PG might be processed more rapidly by phagocytic cells than insoluble staphylococcal cell walls are and ultimately, cleared more rapidly from the body, thus explaining the relatively early onset, the magnitude, and the short duration of the effects of gonococcal PG on temperature and sleep. Alternatively, residual contamination with LPS might have contributed to the biological effects observed. However, precautions were taken to remove LPS during the preparation of gonococcal PG. Furthermore, sleep responses elicited by intravenous injection of LPS are distinguishable from those observed here after gonococcal PG injections. For example, after intravenous LPS injections, enhanced SWS developed within a few minutes (76).

Although the basic chemical structure of bacterial PG is very stable (118), minor structural modifications do occur. For example, the degree of O-acetylation of *S. aureus* cell wall varies

in different growth states (59) or after treatment with antibiotics. Treatment of *S. aureus* (20, 58) or *N. gonorrhoeae* (113) with bacteriostatic agents enhances, while penicillin G decreases, the degree of O-acetylation (1; T. Sidow, L. Johannsen, and H. Labischinski, unpublished observations). A high degree of O-acetylation is correlated with resistance of the staphylococcal (57) and gonococcal cell walls (14, 112) to degradation by lysosomal enzymes and, as shown with staphylococci, to degradation within mammalian macrophages (135). Thus O-acetylation might impair the degradation of cell walls during infection and thereby reduce the availability of MPs capable of triggering immune and sleep responses (23). On the other hand, O-acetylated MPs are more potent somnogens than non-O-acetylated MPs (62). It is, therefore, difficult to predict the effects of cell wall de-O-acetylation on their biological activities. Current results suggest that removal of O-acetyl groups from the staphylococcal cell wall preparation reduced its ability to increase SWS, EEG delta amplitudes, and T_{co}. In contrast, REMS and leukocyte responses were similar whether the O-acetylated preparation or de-O-acetylated preparation was used. Thus removal of the O-acetyl groups differentially affected the biological responses measured, suggesting that the structural requirement for and biological mechanism of these responses are, in part, distinct. Furthermore, it is conceivable that the phosphodiester bond connecting teichoic acid to the C-6 atom of muramic acid (68) is hydrolyzed during the mild alkaline treatment used to de-O-acetylate PG, thereby releasing teichoic acid. The staphylococcal cell wall

preparations used contained both PG and teichoic acid, and teichoic acids have, so far, not been investigated for sleep effects.

About 48% of the muramic acid residues of *N. gonorrhoeae* strain FA19 is O-acetylated, whereas PG from strain RD5 is apparently void of O-acetyl substitutions (126). In contrast to staphylococcal cell walls, different degrees of O-acetylation of the two types of gonococcal PGs did not result in significant differences in biological activity. A structural component that greatly enhances somnogenic potency of monomeric MPs is 1, 6-anhydromuramic acid, which is contained in gonococcal PG (75, 90). Thus any differences in the degree of PG O-acetylation may be inconsequential.

It remains to be determined how bacterial cell walls are processed *in vivo* and whether they are, indeed, transformed into monomeric MPs that reach the brain to alter sleep and other centrally regulated parameters. Cells of the monocyte-macrophage system are known to phagocytize invading bacteria. The ability of a macrophage cell line to digest *Bacillus subtilis* cell walls into monomeric MPs has been demonstrated earlier (134). Also, primary cultures of macrophages can digest *S. aureus* cell walls (135) and preliminary data indicate that macrophages release somnogenic and pyrogenic substances of low molecular weight from phagocytized bacteria (60 and experiment No. 5). Although the role of bacterial cell wall components in normal mammalian physiology remains in debate, current results suggest that these substances

play a pivotal role in the mediation of biological responses during infection.

Experiment No. 5

The results presented in this study suggest that mammalian phagocytic cells produce and secrete somnogenic and pyrogenic muramyl peptides during the digestion of staphylococcal cell walls. The data thus support the hypothesis that the somnogenic muramyl peptides found in mammalian tissues are ultimately derived from the peptidoglycan of bacterial cell walls. The minimal peptidoglycan structure necessary for biological activity, MurNAc-L-Ala-D-iso-Gln (MDP) is contained in the peptidoglycan monomers of most bacteria (118). The structural similarities of those MPs exhibiting biological activity, that is: occurrence of Mur, a short peptide bound via a lactyl ether to the C-3 atom of Mur, and the stereo conformation of alternating L- and D-amino acids, with those found in peptidoglycan has led to the hypothesis that bacterial cell wall peptidoglycan is digested by mammalian cells of the monocyte/macrophage system and that somnogenic MPs are formed and released in the process (23, 63). It has been previously shown that a macrophage cell line is able to digest isolated cell walls of Bacillus subtilis and to consequently release monomeric MPs (134). Also, the ability of primary cultures of bone marrow derived murine macrophages to at least partially digest the insoluble peptidoglycan of *Staphylococcus aureus* has recently been demonstrated (135-136). Here we provide

evidence that some of the resulting digestion products are indeed somnogenic and pyrogenic.

Although we could not provide chemical evidence that the active fractions contained MPs due to the small amount of material available, it is likely that the biological activity of these fractions is attributed to muramyl peptides because: (i) The biological activity coeluted chromatographically with a cell wall specific radioactive marker; (ii) Gel filtration was used to exclude large molecular weight substances, e.g. possible contaminants such as cytokines some of which are pyrogenic and somnogenic; (iii) The samples were heated to 60°C for 30 min to destroy possible cytokine activity; (iv) Using the HPLC system reported, prostaglandins [another class of sleep-active substances (53)] do not elute within the range of fractions collected (data not shown); (v) The lack of sleep promoting activity in most of the samples indicates that the preparatory procedures did not inadvertently introduce contaminating endotoxin because the sleep response of rabbits is very sensitive to endotoxin (21, 76); (vi) The time course of the effects is indistinguishable from those observed after synthetic MPs are injected (69), but unlike those elicited by cytokines (73, 121), endotoxin (21, 76), or prostaglandins E_2 or D_2 (Opp et al., unpublished). For example, results reported here or those elicited by synthetic MPs are characterized by about a hour delay before increases in SWS and decreases in REMS are observed. In contrast, cytokines induce immediate effects (73), endotoxin does not suppress REMS when injected ICV (76), and prostaglandins D_2 or E_2 do not enhance

sleep in rabbits after bolus ICV injections (Opp et al., unpublished); (vii) Samples derived from the control system in which lysozyme degraded isolated bacterial cell walls did not contain cytokines or prostaglandins and elicited sleep responses typical for MPs.

As a result of the separation of the macrophage supernatants by reversed phase HPLC radioactivity of the cell wall specific marker was found distributed over the whole range of the elution gradient indicating that several structurally different MPs were present in the sample. Previously, two synthetic MPs which are contained in the monomeric subunit of *Staphylococcus aureus* peptidoglycan (MDP and MurNAc-L-Ala-D-iso-Gln-γ-L-Lys) were shown to be somnogenic (74). However, of the relatively large number of fractions containing radiolabel only a few contained biological activity. This suggests that either only one of the MPs present in *S. aureus* peptidoglycan is somnogenically active when ICV injected at 10 pmol or that macrophages have the ability to activate and/or inactivate the MPs present.

It is currently unknown whether mammalian enzymes are capable of modifying MPs thereby changing their somnogenic activity. However, previous results concerning MP structure-somnogenic activity relationships suggests several sites at which such modifications could be possible. Thus, modifications of the C-6 of muramic acid, a 1, 6-anhydro structure or a 6-O-acetylation (62), enhance the somnogenic activity of otherwise identical MPs. Further amidation of certain terminal carboxyl groups of some MPs result in the loss of their somnogenic activity (62, 77, 75).

Lysozyme is considered a key enzyme for macrophage degradation of bacterial peptidoglycan. Lysozyme is a muramidase and, thus, capable of producing monomeric MPs. Therefore, we used lysozyme in a cell free system to degrade isolated bacterial cell walls. The low molecular weight products of this digestion process were pyrogenic and somnogenic, thus demonstrating that staphylococcal cell walls contain biological active MPs which can be released by degradation with a mammalian cell wall lytic enzyme.

The common experience that infectious disease is often accompanied by increased sleepiness has recently been experimentally confirmed. Inoculation of rabbits with viable or heat- killed *Staphylococcus aureus* results in complex changes in sleep patterns, initially characterized by increased SWS, decreased REMS, and fever (129). Similar effects are observed when isolated staphylococcal cell walls are intravenously administered (61). The data presented here support the hypothesis that the enhanced sleep and temperature observed during those experiments are, at least in part, due to the release by phagocytic cells of somnogenic and pyrogenic muramyl peptides tailored from bacterial cells walls. The importance of this biological process for normal physiology remains unknown although mammals are constantly exposed to bacteria. Nevertheless, during infection, this process is likely to be important; thus MPs are capable of activating macrophages and the release of MPs by macrophages during the digestion of phagocytized bacteria may lead to a powerful amplification of the immune response.

GENERAL DISCUSSION

Sleep is assumed to result from the structured interactions between cells within the central nervous system (CNS). Cells communicate with each other using two major mechanisms; 1) direct electrical stimulation, (e.g., between heart muscle cells) and 2) chemical stimulation, (e.g., motor neuron cholinergic stimulation of muscle cells). Within the brain the importance of electrical communication between cells remains largely unknown and is not discussed herein. In contrast, much is known about chemical communication. These types of interactions range from very short lasting interactions (msec) confined to very precise anatomical locations, (neurotransmitters), to longer-term influences of a chemical (neuromodulators). Often these latter types of interactions can last minutes and even days (139). Well known examples of neuromodulation within the brain are the actions of certain hormones, [e.g., sex hormones (107)] and growth factors, [e.g., nerve growth factor (105)]. The fundamental tenent of our research is that chemical signals between CNS cells are important for sleep regulation, as evidenced by the basic observations that certain endogenous chemicals (SFs) promote or inhibit sleep when given to normal animals. Indirect evidence also supports the involvement of some putative SFs in sleep regulation. For example, vasoactive intestinal peptide (39), interleukin-1 (IL1) (19) and prostaglandin D_2 (PGD_2) (53) are all located in the preoptic area of the anterior hypothalamus; substances which induce their production (30-31) or substances that they induce production/release of (101) also enhance sleep if

given to normal animals; in the case of IL1, substances that inhibit its production (PGE_2) (80) or actions (alpha-melanocyte-stimulating hormone) (103), inhibit sleep; and IL1 concentrations in CSF vary with sleep/wake cycles (88) (reviewed Annual Report June 1, 1989).

Historically, SFs have been considered to be neuromodulators primarily because their effects on sleep were observed over periods of minutes to several hours rather than msec as in the case of neurotransmitters. However, this distinction is now blurred in that some neurotransmitters influence the production of putative SFs that have long-lasting effects, [serotonin (5HT) can induce astrocyte production of IL1 (122)]. About 30 different endogenous substances have the capacity to alter sleep for periods of one hour or more (reviewed, 71). It is emphasized that some of these substances are more important for sleep than others (71). We have previously presented models that illustrate how SFs interact at both biochemical and neural levels to alter sleep (71). At the biochemical level, SFs may directly, or indirectly via chemical intermediates, alter each others' production or catabolism. For example, glucocorticoids which inhibit sleep (48) inhibit IL1 production (82), whereas other putative SFs that promote sleep, such as muramyl peptides, enhance IL1 production (31). Similarly, the interactions of SFs at the neuronal level may be direct. They could bind to the same receptor complex; thus, vasoactive intestinal peptide and growth hormone releasing hormone may, in rats, enhance slow-wave sleep through a common receptor (101). Alternatively, the interactions of SFs with each

other could be indirect; they could affect different but interconnected neurons. This latter mechanism is likely to occur, but difficult to demonstrate in reference to sleep because it cannot be demonstrated that a given electrophysiological neuronal response is related to sleep regulation.

Regardless of the specific mechanisms of interaction between SFs, current knowledge indicates that neurons are influenced by such factors and that many of these factors influence sleep. This concept differs from the historic postulate of a specific SF with actions that are confined to sleep and that interact specifically with executive sleep centers. This historic concept is no longer tenable for several reasons. First, all putative SFs have biological actions in addition to their ability to alter sleep, and second, the executive sleep center(s) has not been identified despite intense efforts (96). Furthermore, as will be discussed below, sleep may represent a generic property of complex neuronal interactions rather than the product of a specific set of interactions. An important emphasis of SF work is that the neuronal interactions are governed, in a large part, by chemical factors and that this influence may be as important as cellular anatomical connections for sleep regulation. In fact, because some putative SFs are also growth factors, (insulin, IL1), they could dynamically influence neuronal sprouting and hence, anatomical connections.

It is widely postulated that sleep is primarily a neuronal phenomenon, thus our previous models (71) focused on neurons, although a direct role for glia in sleep also seemed likely to us

(70). In our previous model, specific sleep responses resulted from the interactions of multiple SFs with multiple sets of neurons. That model, though useful for presenting the concept of multiple SFs, failed to emphasize that neurons possess dynamic intrinsic and extrinsic properties (see below for examples) that are governed, in part, by SFs and that add greatly to the complexity of neuronal interactions. Specific interactions between neurons are likely to be very different depending on what substances (SFs) are influencing intrinsic properties, and the ongoing neuronal activities that influence extrinsic properties.

With reference to sleep and SFs, these concepts can be illustrated concretely as follows: Many physiological systems affect sleep and in some cases the sensory neurons involved in those systems are altered by IL1 and/or other hormones. For example, sleep is closely linked to temperature regulation (100). IL1 can alter the firing rates of hypothalamic warm and cold sensitive neurons (120). More importantly, some cells that are initially insensitive to changes in temperature become sensitive to heat if exposed to IL1 (37) (i.e., the intrinsic properties of these neurons are dynamic). This observation suggests that IL1 can change the sensory response characteristics of populations of neurons. Moreover, a single neuron can clearly be part of more than one sensory network (55) (extrinsic properties are also dynamic). If IL1 alters responsiveness of sensory neurons, these neuromodulatory actions could create dynamic neuronal networks that regulate sleep via constantly changing individual neuronal components. The term network used in this context refers to the

neuronal interactions *per se* rather than to specific neurons. Depending upon the specific physiological functions driving sleep/wake states (e.g., appetite, temperature, blood pressure, circadian rhythms, etc.) the specific neurons involved and their anatomical location would vary. Under homeostatic conditions, variations in anatomically-relevant sites would be minimal; this would lead to apparent sleep centers. However, during pathological conditions, sleep would still occur, but the specific neurons mediating sleep could be different; (e.g., sleep recovery after brain lesions). Furthermore, substances that affect varied aspects of physiology could influence sleep under some conditions (i.e., when their sensory neurons participate in the sleep network) but not under other conditions. Dynamic changes of electrical intrinsic properties of neurons have previously been recognized as possible mechanisms leading to cyclic variations of vigilance states (81). Although electrical properties of neurons are very important, they are only one (easily measured) of several end products of chemical influences on the neuron.

We propose that animals with complex brains, such as birds and mammals, experience sleep as a result of the activities of different neurons under different conditions. A vast number of lesion experiments support this view to the extent that a recognizable form of sleep eventually reappears regardless of which neurons are removed. If sleep is defined more generally to include behavioral states characterized by "relaxed" postures during periods of immobility, then all animals studied to date exhibit sleep (65). This generalization suggests that sleep could

be considered a generic (basic) property of neuronal interactions, and leads to the question of why neuronal interactions invariably result in sleep/wake cycles. The answers to this question will reveal what sleep does for the brain. Regardless of that answer, it is currently clear that several endogenous substances are capable of altering sleep, that they do so via their effects on CNS cells, and that their interactions are biochemically linked to each other. Thus, the regulation of these substances has become an important facet of the science of sleep regulation.

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TABLE 1. Synthetic IL1α and β peptides tested.

A. IL1α Peptides

115-148	Pro Phe Ser Phe Leu Ser Asn Val Lys Tyr Asn Phe Met Arg Ile Ile Lys Tyr Glu Phe Ile Leu Asn Asp Ala Leu Asn Gln Ser Ile Ile Arg Ala Asn
223-250	Phe Phe Try Glu Thr His Gly Thr Lys Asn Tyr Phe Thr Ser Val Ala His Pro Asn Leu Phe Ile Ala Thr Lys Gln Asp Tyr

TABLE 1. (continued).

B. IL1β Peptides

117-134	Ala Pro Val Arg Ser Leu Asn Cys Thr Leu Arg Asp Ser Glu Glu Lys Ser Leu
178-207	Leu Lys Glu Lys Asn Leu Tyr Leu Ser Cys Val Leu Lys Asp Asp Lys Pro Thr Leu Gln Leu Glu Ser Val Asp Pro Lys Asn Tyr Pro
199-228	Glu Ser Val Asp Pro Lys Asn Tyr Pro Lys Lys Met Glu Lys Arg Phe Val Phe Asn Lys Ile Glu Ile Asn Lys Glu Phe
208-240	Lys Lys Lys Met Glu Lys Arg Phe Val Phe Asn Lys Ile Glu Ile Asn Asn Lys Leu Glu Phe Glu Ser Ala Gln Phe Pro Asn Trp Tyr Ile Ser Thr
247-269	Pro Val Phe Leu Gly Gly Thr Lys Gly Gly Gln Asp Ile Thr Asp Phe Thr Met Gln Phe Val Ser Ser

TABLE 2. Effect of hu-rIL1α and β and several IL1 fragments on rabbit sleep and body temperatures.

							Colonic temperature (°C)		
			NREMS		REMS		T start		T end	
Substance	Dose	N	mean	(SE)	mean	(SE)	mean	(SE)	mean	(SE)
aCSF		7	49.4	(3.3)	10.6	(1.0)	39.2	(0.1)	39.1	(0.1)
IL1α	0.01 ng	7	55.4	(2.5)*	6.2	(0.8)*	39.2	(0.1)	40.4	(0.2)*
IL1α	1.0 ng	7	58.5	(1.9)*	6.0	(0.8)*	39.1	(0.1)	40.5	(0.1)*
IL1α	10.0 ng	7	72.0	(2.1)*	0.8	(0.4)*	39.1	(0.1)	41.6	(0.1)*
aCSF		8	48.6	(2.8)	9.1	(0.8)	39.1	(0.0)	39.5	(0.0)
IL1α heat.	10.0 ng	8	47.8	(2.3)	9.5	(0.9)	39.1	(0.1)	39.4	(0.1)
aCSF		4	55.0	(1.1)	11.0	(0.5)	39.2	(0.1)	39.5	(0.0)
IL1α 115-148	25 µg	4	53.5	(1.0)	9.1	(0.7)	39.3	(0.0)	39.6	(0.1)
aCSF		4	49.1	(3.1)	8.1	(0.2)	39.1	(0.0)	39.3	(0.0)
IL1α 223-250	25 µg	4	47.5	(1.8)	7.2	(0.9)	39.1	(0.1)	39.5	(0.0)
aCSF		8	45.4	(1.9)	8.9	(1.0)	39.1	(0.1)	39.6	(0.1)
IL1β	5 ng	8	53.3	(3.2)*	4.4	(0.8)*	39.2	(0.0)	40.0	(0.1)*
IL1β	20 ng	8	61.5	(4.3)*	2.1	(0.6)*	39.3	(0.1)	41.0	(0.3)*

TABLE 2 (continued)

aCSF		4	50.7	(1.8)	11.9	(2.2)	39.4	(0.1)	39.5	(0.1)
IL1β 117-134	5 µg	4	53.4	(3.1)	9.2	(0.8)	39.2	(0.1)	39.7	(0.1)
aCSF		4	52.3	(2.4)	9.9	(1.6)	39.2	(0.1)	39.3	(0.1)
IL1β 117-134	10 µg	4	48.9	(0.4)	8.6	(2.2)	39.3	(0.1)	39.5	(0.1)
aCSF		7	40.4	(1.8)	5.9	(0.5)	39.2	(0.1)	39.3	(0.1)
IL1β 178-207	5 µg	7	40.5	(2.5)	7.7	(1.1)	39.1	(0.1)	39.5	(0.2)
aCSF		4	55.0	(1.1)	11.0	(0.5)	39.2	(0.1)	39.5	(0.0)
IL1β 178-207	25 µg	4	53.8	(1.9)	10.8	(0.9)	39.4	(0.1)	39.6	(0.0)
aCSF		6	51.6	(2.6)	10.2	(1.1)	39.2	(0.1)	39.3	(0.1)
IL1β 199-228	25 µg	6	50.9	(3.9)	10.0	(1.2)	39.3	(0.1)	39.4	(0.1)
aCSF		9	48.1	(2.0)	8.5	(0.7)	39.2	(0.1)	39.3	(0.1)
IL1β 208-240	25 µg	9	58.8	(3.9)*	5.8	(1.2)	39.1	(0.1)	40.0	(0.2)*
aCSF		4	46.9	(2.8)	9.2	(1.3)	39.2	(0.1)	39.4	(0.1)
IL1β 247-269	1 µg	4	45.6	(1.9)	9.3	(1.6)	39.4	(0.1)	39.7	(0.1)
aCSF		3	44.8	(3.2)	8.2	(1.3)	39.4	(0.0)	39.5	(0.1)
IL1β 247-269	5 µg	3	46.5	(2.3)	9.8	(1.8)	39.4	(0.1)	39.7	(0.1)
aCSF		3	45.0	(2.4)	8.1	(0.7)	39.3	(0.0)	39.4	(0.1)
IL1β 247-269	10 µg	3	47.7	(2.3)	7.9	(0.8)	39.2	(0.1)	39.5	(0.1)

Abbreviations used in table: aCSF = artificial CSF; IL1α heat. = heat-inactivated IL1α; *Indicates significant difference between values obtained after administration of test substances and corresponding values from same rabbits obtained after injection of aCSF as control. Friedman's test was used for vigilance states; colonic temperatures were evaluated by means of wilcoxon tests.

TABLE 3. Average EEG Delta Wave Amplitudes (μV) during NREMS

Injectant	aCSF	IL1-α 0.01 ng	IL1α 1.0 ng	IL1-α 10 ng
Hour 1	124.8±10.7	144.6±13.9*	150.2±15.8*	152.2±17.3*
Hour 2	127.5±11.8	139.0±14.6*	145.0±16.2*	157.0±15.9*
Hour 3	125.3±13.2	142.5±16.2*	139.9±14.4*	147.9±18.2*
Hour 4	122.3±11.2	139.4±12.9*	140.8±15.2*	141.3±11.2*
Hour 5	124.3±11.3	138.6±14.4*	132.4±10.3*	144.8±14.0*
Hour 6	126.2±13.8	133.4±13.3	132.6±12.4	138.1±13.4*

+ Values are means ± SE

* Indicates significant difference; Friedman's test across doses indicated significant dose-dependent effect.

TABLE 4. Effect of IL1α and β synthetic peptides on PGE_2 production by fibroblasts and proliferation of T cells.

		[3H] Thymidine Incorporation (cpm ± SEM)**	
		Experiment 1	Experiment 2
	PGE_2*	(Murine Thymocytes)	(Murine D10 Cells)
IL1α Peptide			
115-148	BDL (1-25)+	---	1,073 ± 39 (1)+
223-250	63 ± 7 (12.5)+	---	542 ± 42 (1)+
IL1β Peptide			
117-134	---	---	661 ± 33 (50)+
178-207	13 ± 2 (50)+	954 ± 86 (100)+	---
199-228	---	---	2,799 ± 652 (100)+
208-240	105 ± 18 (50)+	107 ± 16 (100)+	---
247-269	---	---	3,179 ± 531 (50)+
PBS	BDL	102 ± 10	478 ± 47

*PGE levels measured after 48-h exposures of adherent RA synovial cells in serum-free MEM; values one mean ± SD in ng/ml. The detection limit was 10/ng/ml; BOL is below detection limit.

+Numbers in brackets are μg of peptides/ml tested (see Methods).

**In Experiment 1, target cells were murine thymocytes, and in Experiment 2 murine D10 T cell line.

TABLE 5. Effects of 1 nmol/kg 3'-5'CP (sample 1: before HPLC purification, sample 2: after HPLC purification) on the mean (±S.E.M.) percent time spent in the various states of vigilance (W: wakefulness; NREMS: non-REM sleep; REMS: rapid eye movement sleep) during a 6-h recording period. Asterisks denote significant differences (Wilcoxon matched pairs signed ranks test; $p < 0.05$).

	Sample 1 (n=8)		Sample 2 (n=7)	
	aCSF	3'-5'CP	aCSF	3'-5'CP
W	44.8 ± 2.0	33.7 ± 1.6*	44.8 ± 2.5	37.0 ± 2.2*
NREMS	49.8 ± 1.7	60.2 ± 1.6*	47.3 ± 2.2	52.6 ± 2.0*
REMS	5.5 ± 0.6	6.1 ± 0.9	7.8 ± 0.9	10.3 ± 1.2

TABLE 6. Effects of saline injection on rectal temperature and hematologic parameters in rabbits

Time, h	Temp, °C	WBC, % control	Neutr, %WBC	Lymph, %WBC	nRBC, no./100 WBC	Cortisol, µg/dl	Trigly, mg/dl	Glucose, mg/dl
0	38.5±0.1	100±6	28±3	68±3	0.1±0.1	6.5±0.5	140±23	138±2
6	38.7±0.1	89±4	40±3*	58±3	0.6±0.3	8.2±0.8	106±8	139±5
12	38.6±0.1	94±3	39±3	58±3	0.5±0.4	7.1±0.4	127±28	136±3
24	38.5±0.1	107±4	35±4	61±4	0.1±0.1	5.4±0.3	152±28	139±5
36	38.7±0.1	92±5	34±5	61±4	1.2±0.9	7.0±0.7	133±15	142±3
48	38.6±0.1	106±6	31±2	65±2	0.8±0.6	5.4±0.4	174±30	142±4

Values are means ± SE; n = 8 (2 animals were injected via each inoculatin route, and data were pooled). Temp, rectal temperature; WBC, white blood cells; Neutr, neutrophilis; Lymph, lymphocytes; nRBC, nucleated red blood cells; Trigly, triglycerides. Mean no. of WBC present at time 0 was 6,779±417 cells/µl. *$P<0.05$ relative to time 0.

TABLE 7. Effects of intravenous inoculation with *P. multocida* on rectal temperature and hematologic parameters in rabbits

Time h	Temp, °C	WBC, % control	Neutr, %WBC	Lymph, %WBC	nRBC, no./100 WBC	Cortisol, µg/dl	Trigly, mg/dl	Glucose, mg/dl
0	38.4±0.2	100±8	24±3	72±3	1.0±0.4	6.0±0.5	142±29	144±3
6	39.8±0.2*	58±8*	76±3*	23±3*	26.8±12.0*	13.5±1.9*	170±36	145±9
12	39.5±0.3*	120±18	75±4*	23±4*	16.2±7.5*	11.3±1.9	312±124	128±5*
24	39.8±0.4*	78±14	71±7*	26±7*	23.2±12.3*	19.7±4.8*	965±356*	142±12

Values are means ± SE; n = 8. Temp, rectal temperature; WBC, white cells; Neutr. Neutrophils; Lymph, lymphocytes; nRBC, nucleated red blood cells; Trigly, triglycerides. Mean no. of WBC present at time 0 was 8,698 ± 672 cells/µl. *$P < 0.05$ relative to time 0.

Table 8. Bacteriologic culturing of tissue samples from rabbits challenged with *P. multocida*

Route of Inoculation	Positive Samples/Total Samples	
	Blood	Pulmonary lavage
iv	5/5	ND
im	2/4	ND
in	2/10	9/10
sc	2/6	ND

All tissue samples were collected after animals were killed. Rabbits challenged via iv route were killed 24 h after challenge. Rabbits challenged via other routes were killed 48 h after challenge. ND, not determined.

Table 9. Effects of cell wall preparations on rabbit SWS, EEG delta voltage, REMS, and T_{co}

	Dose, mg	n	%SWS		EEG Delta Voltage		%REMS		T_{co} (°C), 6 h	
			Control	Expt	Control	Expt	Control	Expt	Control	Expt
S. aureus cell wall	0.9	7	48±1	54±3†	87±6	99±8†	8.4±1.3	3.8±1.0†	39.2±0.1	39.9±0.2*
	2.4	8	49±1	60±2†	100±5	121±7†	7.8±1.2	3.0±0.4†	39.3±0.1	40.0±0.1*
S. aureus cell wall de-*O*-acetylated	0.9	8	50±2	53±3	78±12	85±15	5.0±1.0	2.2±0.6†	39.5±0.1	39.9±0.2
	2.4	8	47±3	58±4†	90±17	110±21†	5.3±0.9	3.0±0.7†	39.4±0.1	40.3±0.1*
N. gonorrhoeae S-*O*-PG	0.5	4	49±1	53±2	108±5	139±12	4.6±0.4	1.2±0.4†	39.3±0.0	39.5±0.2
N. gonorrhoeae S-non-*O*-PG	0.5	4	42±3	57±4†	85±24	119±35†	3.2±0.4	0.6±0.2†	39.4±0.0	39.6±0.2

Values are means ± SE. Sleep values are 6-h postinjection averages; n, no. of rabbits; SWS, slow-wave sleep; EEG delta voltage, average electroencephalographic delta-wave voltage (μV); REMS, rapid-eye-movement sleep; T_{co}, colonic temperature 6-h postinjection; S-*O*-PG, strain FA19 soluble peptidoglycan; S-non-*O*-PG, strain RD5 soluble peptidoglycan; $n = 7$ for EEG delta voltages of *S. aureus* de-*O*-acetylated cell wall. † $P < 0.05$ Friedman test. * $P < 0.05$ Wilcoxon matched-pairs signed ranks test.

Table 10. Effects of cell wall preparations on rabbit lymphocyte and neutrophil numbers (as percent of WBC)

	Dose, mg	*n*	Lymphocytes, %WBC				Neutrophils, %WBC			
			Control		Experiment		Control		Experiment	
			Postinjection h		Postinjection h		Postinjection h		Postinjection h	
			0	6	0	6	0	6	0	6
S. aureus cell wall	0.9	4	69±4	67±2	70±1	38±4*	28±4	31±2	25±2	59±4*
	2.4	8	69±2	58±4	66±1	23±2*	28±2	38±3	30±1	73±3*
S. aureus cell wall de-*O*-acetylated	0.9	4	63±3	64±3	64±4	36±5*	33±4	32±3	33±3	61±6*
	2.4	8	67±3	63±2	67±2	32±5*	29±3	33±2	30±2	67±6*
N. gonorrhoeae, S-*O*-PG	0.5	4	69±2	50±3	62±5	29±8*	28±2	46±5	33±4	77±5*
N. gonorrhoeae, S-non-*O*-PG	0.5	4	70±4	63±2	77±5	28±3*	25±4	34±2	23±5	72±2*

Values are means ± SE; *n*, no. of rabbits; WBC, white blood cells; S-*O*-PG, strain FA19 peptidoglycan; S-non-*O*-PG, strain RD5 peptidoglycan.
* $P < 0.05$, Wilcoxon matched-pairs signed ranks test; experimental values compared with control values taken at same time of day from same rabbits.

Table 11. Effects of enzymatic digestion of *Staphylococcus aureus* cells and isolated cell walls on rabbit sleep and temperature. Sample 1 was produced by digestion of intact staphylococci by murine macrophages, samples 2 and 3 were produced by a cell free system in which lysozyme degraded isolated de-O-acetylated staphylococcal cell walls. The digestion products were separated by gel filtration chromatography and fractions were pooled as indicated. Fraction numbers refer to Figure 1. From the radioactivity in a sample, the content of muramic acid was calculated. Aliquots were injected ICV into rabbits and EEG, movement, and brain temperature were monitored for 6 hours during daytime. Controls were injected with vehicle (artificial CSF) alone. Slow-wave sleep (SWS), rapid-eye-movement sleep (REMS), and δ-wave amplitudes are means of 6 hours ± SEM. Colonic temperatures are temperatures at the end of the recording period. n, Number of animals. *, $P < 0.02$; **, $P < 0.01$; Wilcoxon matched-pairs signed ranks test; + significant (≥0.5°C) increases in colonic temperature. Experimental values were compared with control values taken at the same time of day from same rabbits. $, only h 2-6 were included in the calculation; nd, not determined

#	digested by	fractions pooled	dose pmol Mur	n	SWS [% of time] control	SWS [% of time] exper.	REMS [% of time] control	REMS [% of time] exper.	δ wave amplitudes control	δ wave amplitudes exper.	Colonic temp. [°C] control	Colonic temp. [°C] exper.
1	macrophage	20-38	350	8	44±2	57±3**	3.9±0.6	1.6±0.5**	77±8	97±7**	39.3±0.1	41.1±0.2+
2	lysozyme	8-22	2000	4	58±3	72±6**	7.0±0.5	3.7±1.3*	nd	nd	39.4±0.1	40.9±0.2+
3	lysozyme	23-37	630	4	51±4	65±9**$	9.5±0.4	1.0±0.8**$	nd	nd	39.5±0.0	40.6±0.3+

Table 12. Effects on rabbit sleep and colonic temperature of components of macrophage supernatants after digestion of staphylococci and purification by gel filtration and reversed phase chromatography. Low molecular weight digestion products of the macrophage assay obtained from gel filtration chromatography were fractionated by HPLC and fractions were pooled as indicated in Figure 19. Aliquots were injected ICV into rabbits and EEG and brain temperature were monitored for 6 hours. The amount of sample injected was determined from the contents of ^{14}C- radioactivity. The amount injected depended, in part, on the availability of sample. Controls were injected with vehicle (artificial CSF) alone. Slow-wave sleep (SWS), rapid-eye-movement (REMS), and δ-wave amplitudes are means of 6 hours ± SEM. Colonic temperatures are temperatures at the end of the recording period. n, number of animals. *P < 0.02; **P < 0.01; Wilcoxon matched-pairs signed ranks test; + significant (≥ 0.5°C) increase in colonic temperature; experimental values were compared with control values taken at the same time of day from same rabbits.

# exper.	fractions pooled	dose pmol Mur	n	SWS [% of time] exper.	SWS [% of time] control	REMS [% of time] exper.	REMS [% of time] control	δ-wave amplitudes exper.	δ-wave amplitudes control	Colonic temperatur exper.	
1	2, 4	10	4	57±1	56±1	8.6±1.2	8.7±1.0	99±7	96±1	39.4±0.1	39.3
2	6- 9	50	4	56±2	55±2	8.4±0.8	5.4±0.8	96±7	102±10	39.3±0.0	39.7
3	10-12	10	4	57±1	55±2	8.6±1.2	9.9±1.3	99±7	98±8	39.4±0.1	39.4
4	13,14	10	4	48±2	50±2	8.7±1.3	7.9±0.6	74±11	74±9	39.3±0.1	39.3
5	15-16	10	4	59±2	59±2	8.5±1.2	9.4±1.6	108±6	108±6	39.5±0.1	39.8
6	18	10	4	57±3	57±2	10.9±0.8	9.5±0.5	109±4	107±5	39.4±0.1	39.5
7	20-22	50	4	48±3	50±2	6.6±0.6	5.7±0.4	65±7	72±6	39.3±0.1	40.3
8	24-26	10	4	52±2	65±4**	9.4±0.9	4.7±0.7**	79±11	100±9**	39.3±0.2	40.7
		50	4	57±1	70±2**	8.6±1.2	2.6±0.8**	99±7	121±10**	39.4±0.1	41.2
9	28-30	10	3	55±3	54±2	10.8±1.1	10.5±1.9	107±5	112±8	39.3±0.1	39.5
10	31,32	10	4	52±2	53±2	9.4±0.9	9.4±1.0	79±11	78±9	39.3±0.2	39.1
11	34	10	4	52±2	52±2	9.4±0.9	7.9±1.5	79±11	87±8	39.3±0.2	39.4
12	36-39	50	4	56±2	56±2	8.4±0.8	5.2±0.8	96±7	104±10	39.3±0.0	40.5
13	41	10	4	48±2	48±2	8.7±1.3	9.3±1.0	74±11	71±7	39.3±0.1	40.0
14	43-47	10	4	57±3	52±2	10.9±0.8	8.4±1.7	109±4	105±7	39.4±0.1	39.9
		50	4	48±3	61±1*	6.5±0.6	4.6±1.6	65±7	84±9**	39.3±0.1	41.3
15	50,51	10	6	53±2	55±3	10.5±0.7	8.1±1.2	94±7	116±14*	39.2±0.1	39.9
16	55,56	10	4	55±0	49±2	9.7±0.9	7.4±0.7	75±7	78±7	39.2±0.1	39.6
17	58-61	50	4	48±2	51±3	8.7±1.3	8.4±0.8	74±11	74±11	39.3±0.1	39.7
18	68-70	10	3	54±2	54±2	10.5±1.7	6.2±1.9	100±7	101±11	39.4±0.1	39.2
19	78	10	4	52±2	49±2	9.6±0.9	9.1±0.5	71±9	73±8	39.3±0.1	39.0

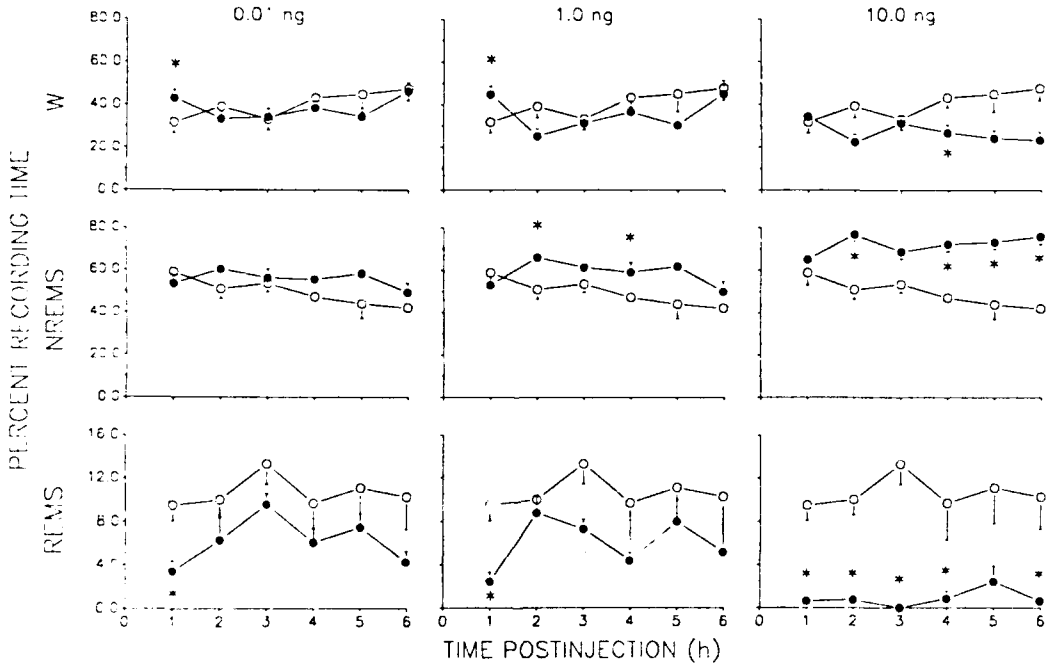

Fig. 1. Effects of 0.01 (left), 1.0 (middle) and 10 ng (right) of interleukin-1α (IL1α) on rabbit vigilance states. Each point represents the hourly mean percent of time spent in wakefulness (W), non-rapid-eye movement sleep (NREMS) or rapid-eye movement sleep (REMS). Control values are from same group (n = 7) of rabbits obtained after injection of vehicle. IL1α dose-dependently enhanced NREMS and inhibited REMS.

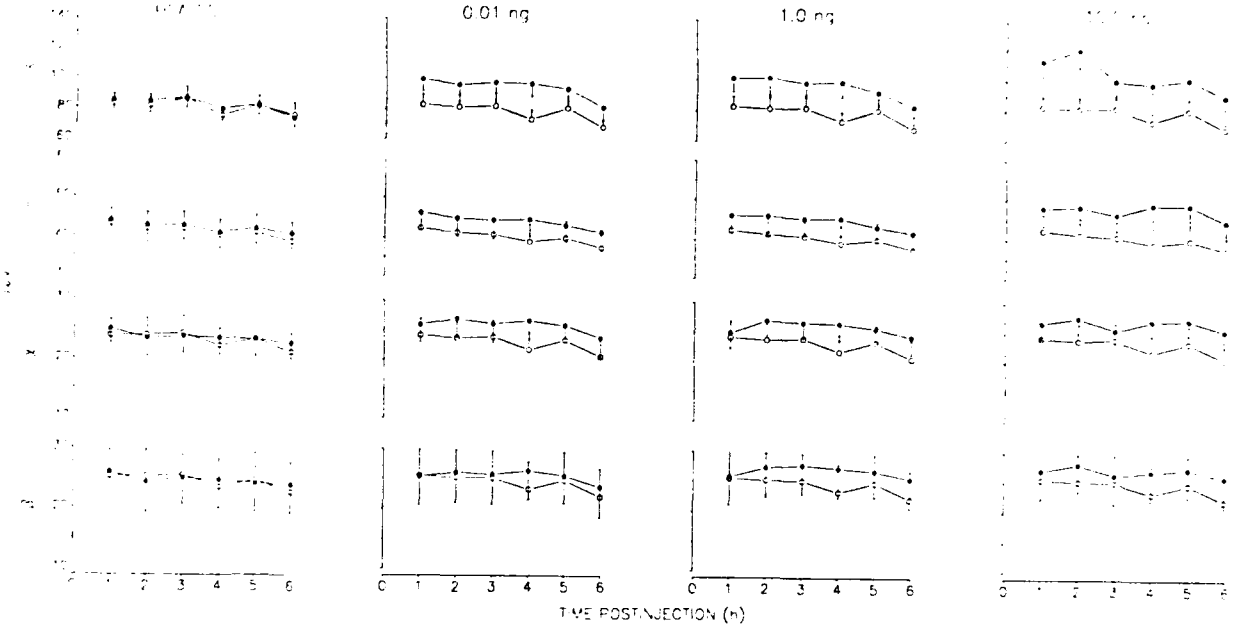

Fig. 2. Effects of heat-treated IL1α and three doses (0.01, 1.0 and 10.0 ng) of IL1α on hourly average electroencephalographic (EEG) voltages. Each point represents the mean ± SE (n = 7) voltage for each hour in the EEG delta (0.5-3.5 Hz), theta (4-7.5 Hz), alpha (8-12.5 Hz) and beta (13-25 Hz) frequency bands. Control values are from same rabbits after vehicle injections. IL1α dose-dependently enhanced EEG delta waves. The highest dose of IL1 also significantly enhanced EEG theta waves. EEG alpha and beta waves were unaffected by IL1α. Heated treated IL1α was completely inactive.

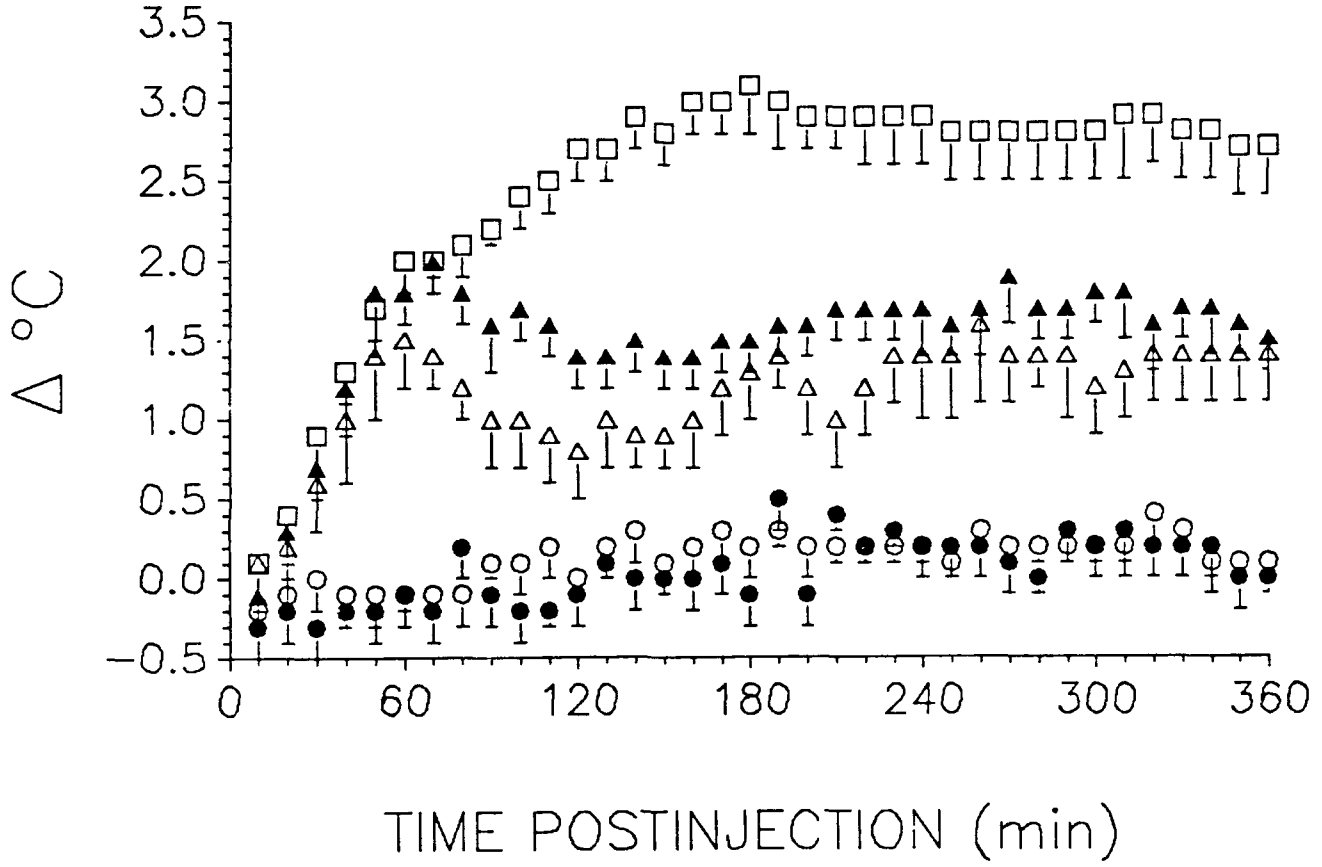

Fig. 3. Time courses of brain temperature (T_{br}) after intracerebroventricular injection of interleukin-1α (IL1α) (0.01Δ; 1.0▲ and 10.0 ⊐ ng), heat treated IL1α (●) or vehicle (○). Values are means ± SE for 7 rabbits; each value was determined every 10 min throughout the assay period. IL1α dose-dependently enhanced brain temperatures; this activity was destroyed by heat-treating (75°, 30 min.) IL1α prior to injection.

GHRH	N-t	**His**	**Ala**	**Asp**	**Ala**	**Ile**	**Phe**	**Thr**	**Ser**	**Ser**	**Tyr**
+RNA	5'	CAU	GCA	GAC	GCC	AUC	UUC	ACC	AGC	AGC	UAC
-RNA	3'	GUA	CGU	CUG	CGG	UAG	AAG	UGG	UCG	UCG	AUG
3'→5' CP	N-t	**Val**	**Arg**	**Leu**	**Arg**	**STOP**	**Lys**	**Trp**	**Ser**	**Ser**	**Met**
5'→3' CP	C-t	**Met**	**Cys**	**Val**	**Gly**	**Asp**	**Glu**	**Gly**	**Ala**	**Ala**	**Val**

GHRH	**Arg**	**Arg**	**Ile**	**Leu**	**Gly**	**Gln**	**Leu**	**Tyr**	**Ala**	**Arg**
+RNA	CGG	AGA	AUC	CUG	GGC	CAA	UUA	UAU	GCC	CGC
-RNA	GCC	UCU	UAG	GAC	CCG	GUU	AAU	AUA	CGG	GCG
3'→5' CP	**Ala**	**Ser**	**STOP**	**Asp**	**Pro**	**Val**	**Asn**	**Ile**	**Arg**	**Ala**
5'→3' CP	**Pro**	**Ser**	**Asp**	**Gln**	**Ala**	**Leu**	**STOP**	**Ile**	**Gly**	**Ala**

GHRH	**Lys**	**Leu**	**Leu**	**His**	**Glu**	**Ile**	**Met**	**Asn**	**Arg**	**Gln**
+RNA	AAA	CUG	CUG	CAC	GAA	AUC	AUG	AAC	AGG	CAG
-RNA	UUU	GAC	GAC	GUG	CUU	UAG	UAC	UUG	UCC	GUC
3'→5' CP	**Phe**	**Asp**	**Asp**	**Val**	**Leu**	**STOP**	**Tyr**	**Leu**	**Ser**	**Val**
5'→3' CP	**Phe**	**Gln**	**Gln**	**Val**	**Phe**	**Asp**	**His**	**Val**	**Pro**	**Leu**

GHRH	**Gln**	**Gly**	**Glu**	**Arg**	**Asn**	**Glu**	**Glu**	**Gln**	**Arg**	**Ser**
+RNA	CAA	GGG	GAG	AGG	AAC	CAG	GAA	CAA	AGA	UCC
-RNA	GUU	CCC	CUC	UCC	UUG	GUC	CUU	GUU	UCU	AGG
3'→5' CP	**Val**	**Pro**	**Leu**	**Ser**	**Leu**	**Val**	**Leu**	**Val**	**Ser**	**Arg**
5'→3' CP	**Leu**	**Pro**	**Leu**	**Pro**	**Val**	**Leu**	**Phe**	**Leu**	**Ser**	**Gly**

GHRH	**Arg**	**Phe**	**Asn**	**C-t**
+RNA	AGG	UUC	AAC	3'
-RNA	UCC	AAG	UUG	5'
3'→5' CP	**Ser**	**Lys**	**Leu**	**C-t**
5'→3' CP	**Pro**	**Glu**	**Val**	**N-t**

Fig. 4. The primary structure of the 3'-5' CP of rGHRH as derived from the nucleotide sequence of the messenger RNA of rGHRH (+mRNA). The complementary mRNA (-mRNA) is aligned antiparallel with the +mRNA. As the translation of the -mRNA was carried out in the 3' to 5' direction, rGHRH and the 3'-5' CP are aligned parallel. The numbering of the amino acids in the 3'-5' CP starts at the N-terminal (N-t) of the synthesized peptide. The STOP codons, the synthesized 3'-5' CP and the corresponding sequence of rGHRH are shown in bold. Fig. 4 does not show that the C-terminal (C-t) Leu of the synthesized 3'-5' CP is amidated.

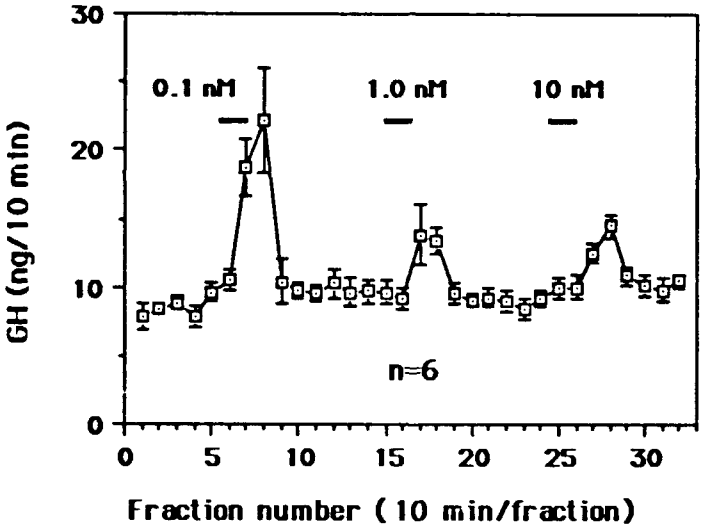

Fig. 5. Stimulation of GH secretion from perifused adult male rat pituitary cells. The values are means ± S.E.M. (n = 6). The horizontal bars denote the time of exposure to the 3'-5' CP (10 min).

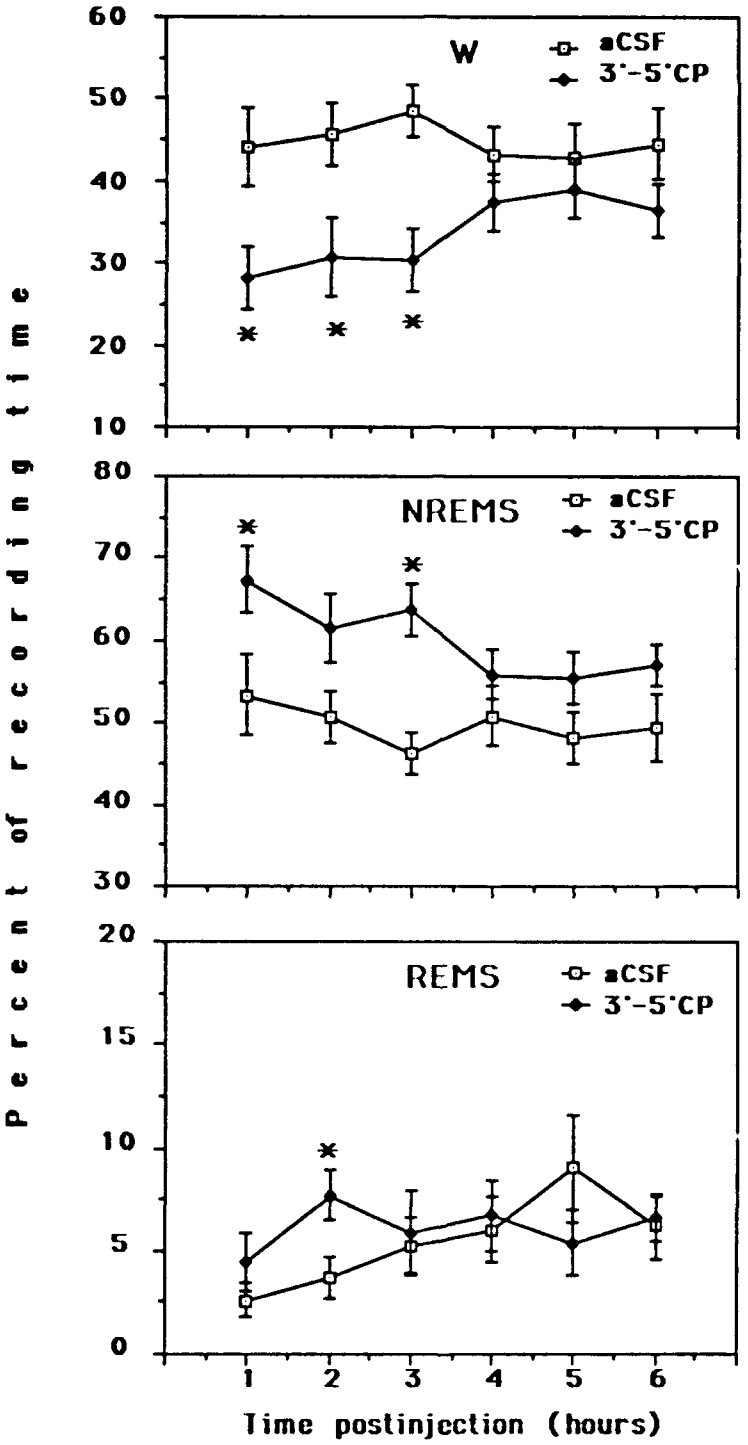

Fig. 6. Effects of icv injection of aCSF and 1 nmol/kg 3'-5' CP on rabbit sleep-wake activity. Percent of time (mean ± S.E.M.) spent in wakefulness (W), NREMS and REMS are presented for postinjection hours 1 to 6. Asterisks denote significant differences (Wilcoxon matched pairs of signed ranks test; $p < 0.05$.).

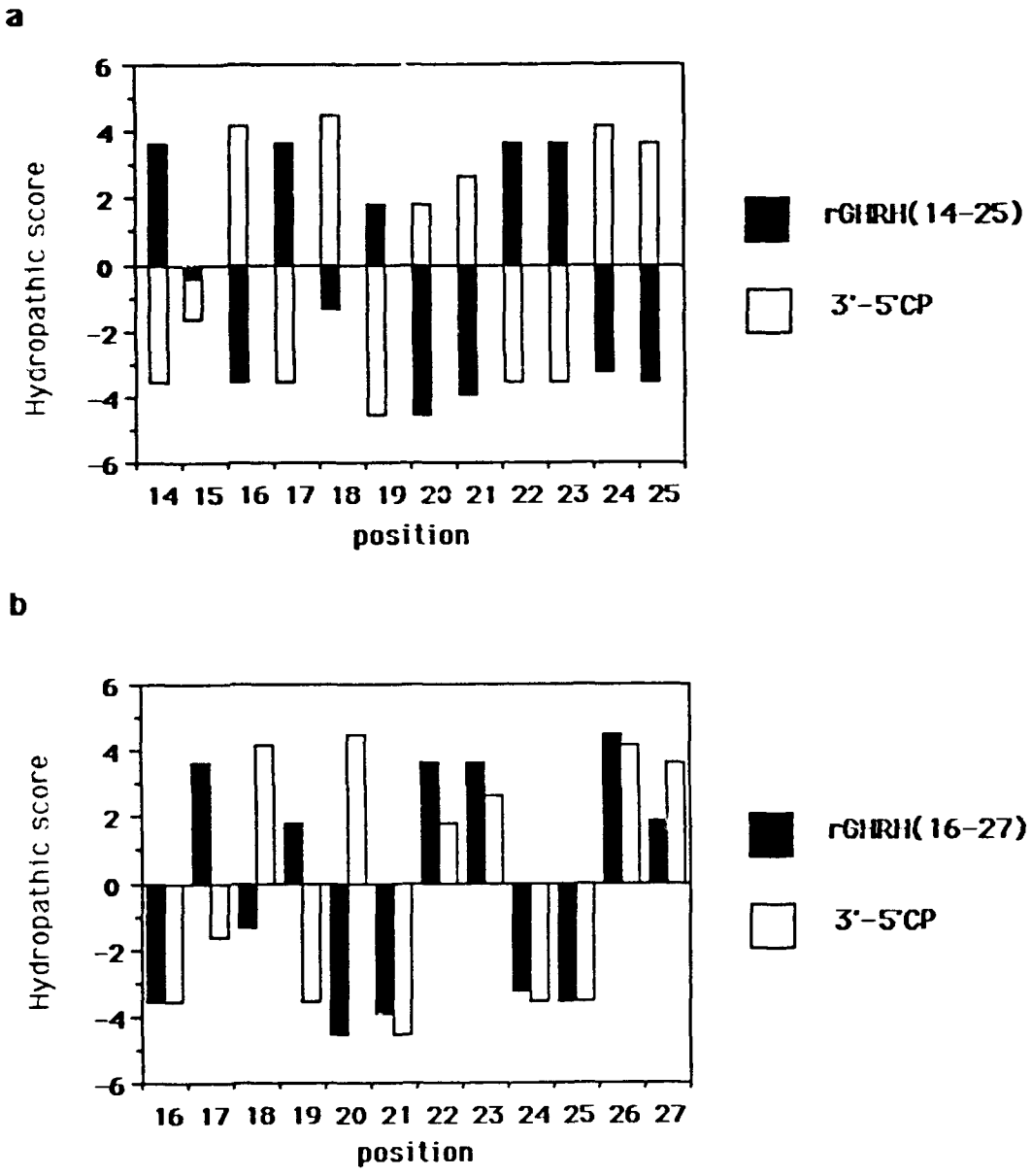

Fig. 7. Individual hydropathic scores of the amino acids of rGHRH (14-25) and the 3'-5' CP (a), and those of rGHRH (16-27) and the 3'-5' CP (b) according to the scale of Kyte and Doolittle. The peptides are aligned parallel. The numbers at the bottom denote the positions of the amino acids in rGHRH. When the peptides are aligned keeping their relative positions (a), significant ($p < 0.005$) anticomplementarity occurs in their hydropathic scores ([CP] = -0.966 [rGHRH] - 0.215, $r = 0.891$). After shifting the CP-sequence by two amino acids (about half turn of the alpha helix) towards the C-terminal of rGHRH (b), the terminal heptapeptides (rGHRH [21-27] and 3'-5' CP [6-12]) show significant ($p < 0.005$) complementarity in their hydropathic scores ([CP] = 0.951 [rGHRH] - 0.306, $r = 0.956$).

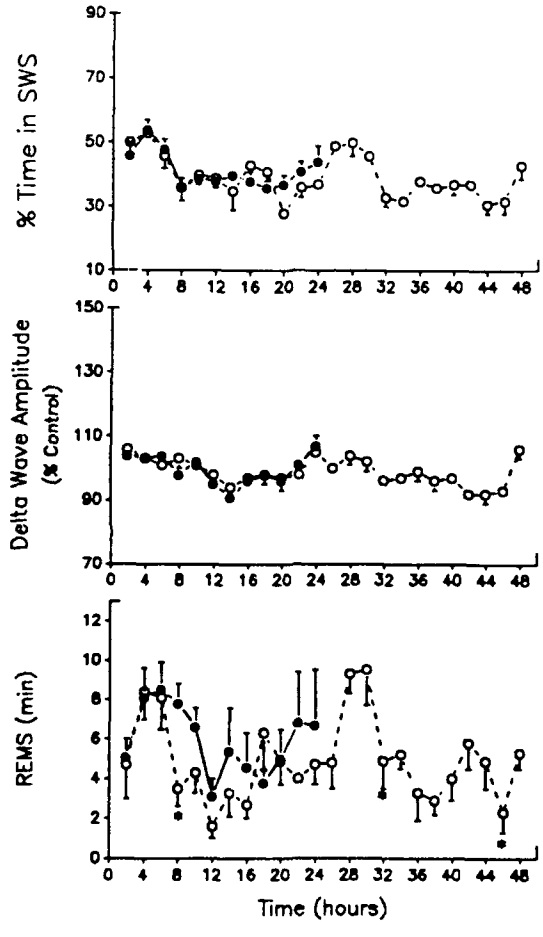

Fig. 8. Sleep patterns in rabbits after inoculation with sterile pyrogen-free saline. Slow-wave sleep (SWS), δ-wave amplitude (DWA), and rapid-eye-movement sleep (REMS) were monitored in rabbits for 24 h before (●—●) and 48 h after (○---○) administration of 0.7 ml of sterile pyrogen-free saline via iv, im, in, or sc routes (n = 2 for each route). Lights were off during hours 11-23 and 35-47. Data points represent means ± SE of values obtained during 2-h intervals from 8 rabbits. *P < 0.05 relative to corresponding base-line values.

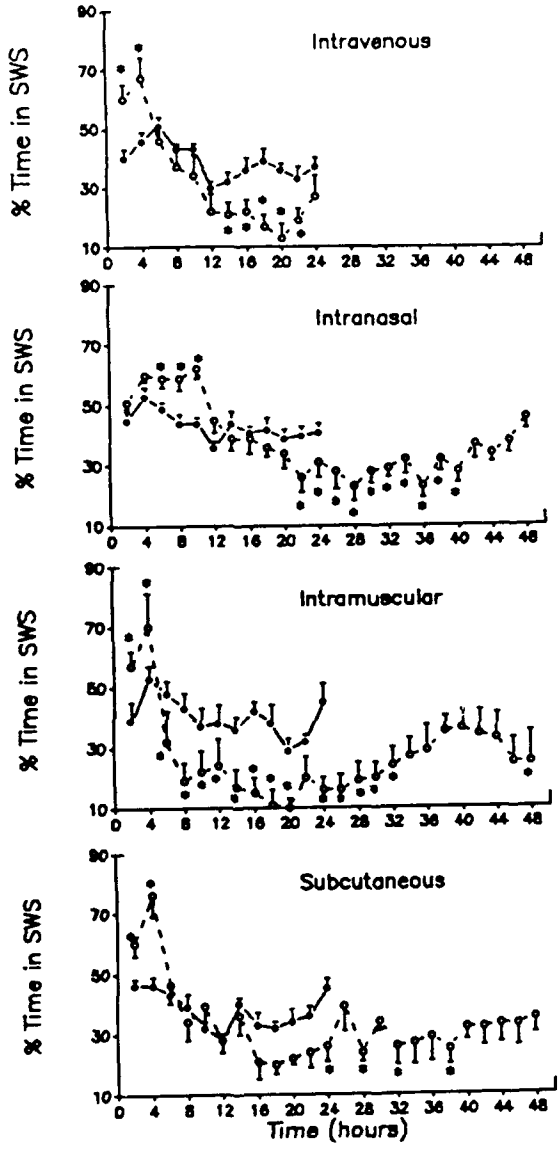

Fig. 9. SWS in rabbits after inoculation with *P. multocida*. SWS was monitored for 24 h before (●—●) and 24-48 h after (O---O) inoculation of rabbits with viable *P. multocida*. Following routes of administration, doses, and no. of rabbits were used; iv, $6.7 \pm 1.2 \times 10^7$ CFU, n = 8; im, $8.2 \pm 0.4 \times 10^8$ CFU, n = 6; in, $9.6 \pm 1.2 \times 10^8$ CFU, n = 10; sc, $2.9 \pm 0.2 \times 10^9$ CFU, n = 6. Lights were off during hours 11-23 and 35-47. Data points represents means ± SE of values obtained during 2-h intervals. *P < 0.05 relative to corresponding base-line values.

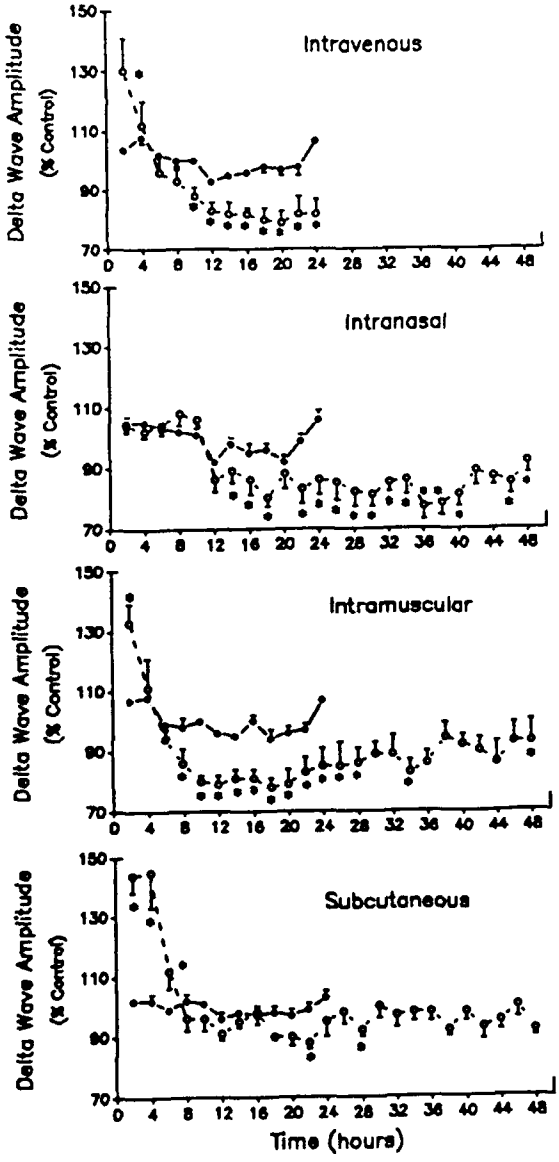

Fig. 10. DWA in rabbits after inoculation with *P. multocida*. DWA was monitored for 24 h before (●—●) and 24-48 h after (○---○) inoculation of rabbits with viable *P. multocida* as described in Fig. 9. Data points represent means ± SE of values obtained during 2-h intervals. *P < 0.05 relative to corresponding baseline values.

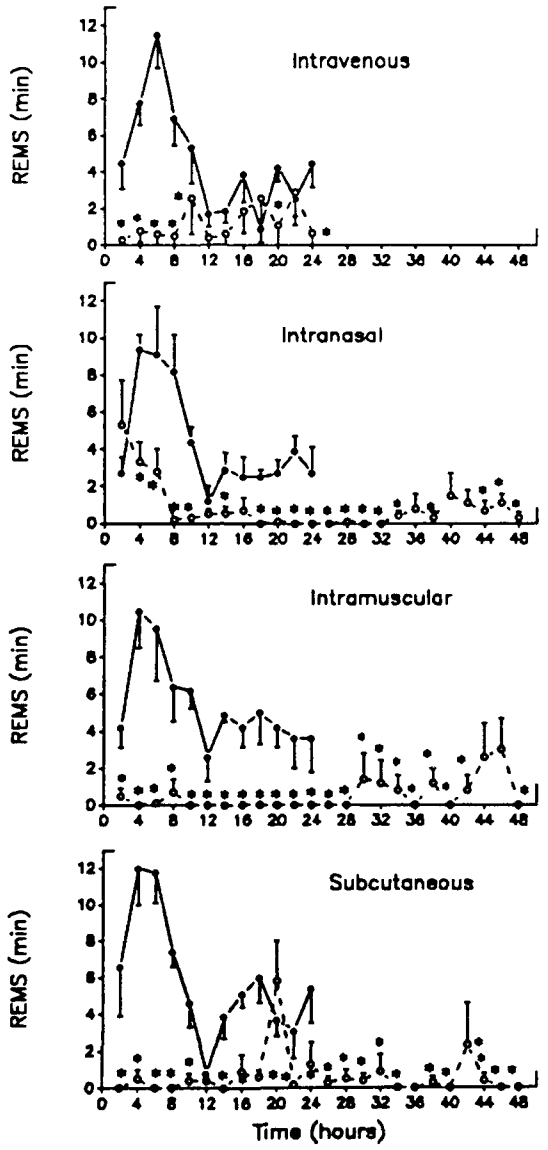

Fig. 11. REMS in rabbits after inoculation with *P. multocida*. REMS was monitored for 24 h before (●—●) and 24-48 h after (○---○) inoculation of rabbits with viable *P. multocida* as described in Fig. 9. Data points represent means ± SE of minutes of REMS that occurred during 2-h intervals. *P < 0.05 relative to corresponding base-line values.

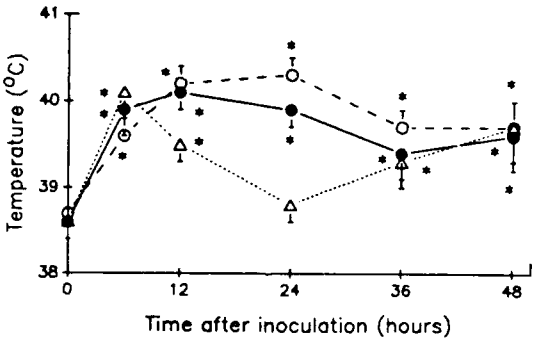

Fig. 12. Rectal temperatures of rabbits after inoculation with *P. multocida.* Rabbits were inoculated with *P. multocida* as described in Fig. 9. Rectal temperatures and blood samples were obtained before (time 0) and every 6-12 h after inoculation. Data are expressed in °C. Data points represent means ± SE. , im; O---O, in; Δ---Δ, sc. *P < 0.05 relative to time 0.

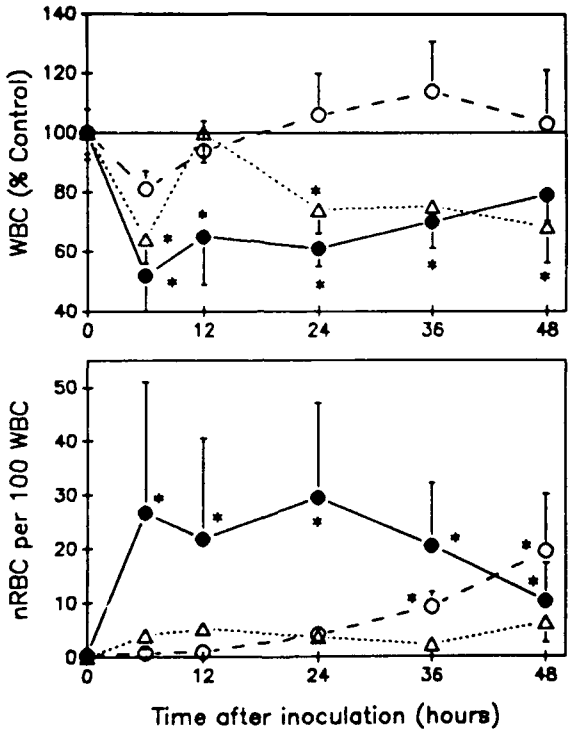

Fig. 13. Total WBC count and nRBC in rabbits after inoculation with *P. multocida*. Rabbits were inoculated with *P. multocida* as described in Fig. 9. Mean ± SE of WBC at time 0 in rabbits inoculated via in, im, and sc routes were 7,460 ± 585, 8,007 ± 694, and 7,589 ± 536 cells/µl, respectively. All total WBC counts were corrected for nRBC, if present. Data are expressed as percentage of preinoculation values (time 0). Data points represent means ± SE. ●——● , im; ○---○, in; Δ---Δ, sc. *P < 0.05 relative to time 0.

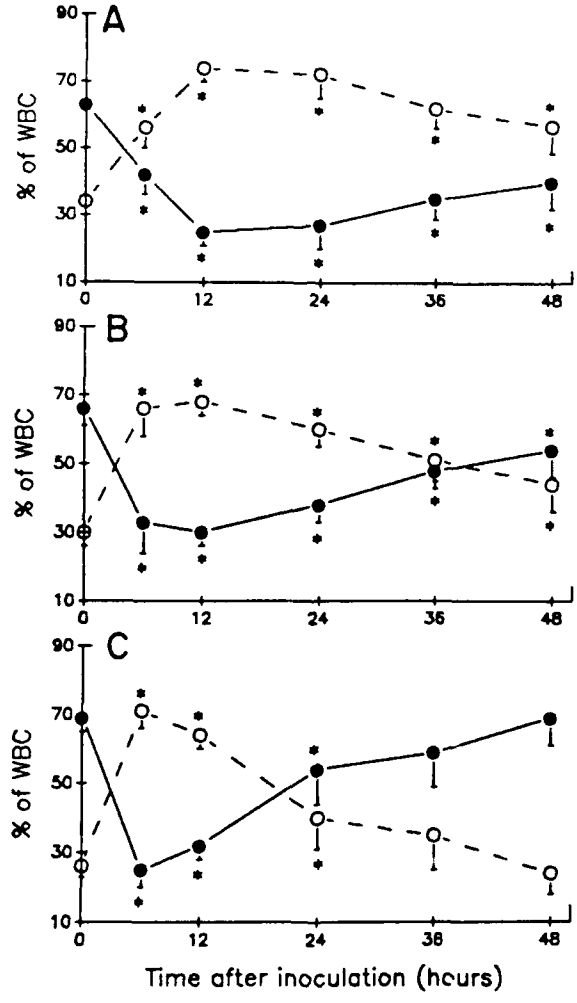

Fig. 14. WBC distribution in rabbits after inoculation with *P. multocida*. Rabbits were inoculated with *P. multocida* as described in Fig. 9. A, B, and C represent effects of in, im, and sc inoculation, respectively. Data are expressed as percentage of individual cell types/100 WBC. Data points represent means ± SE. O---O, neutrophils; ●—● , lymphocytes. *P < 0.05 relative to time 0.

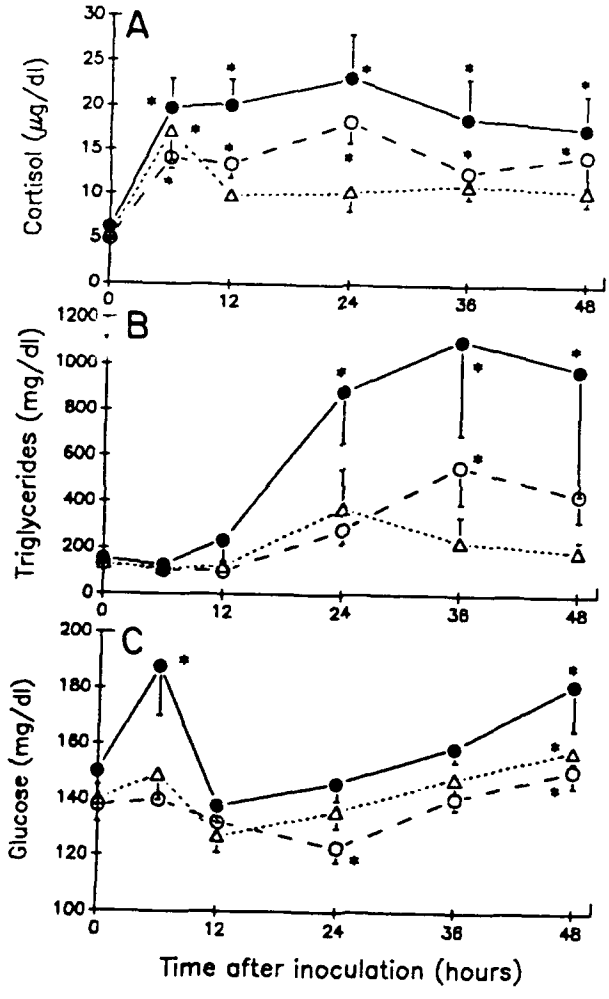

Fig. 15. Plasma concentrations of cortisol, triglycerides, and glucose in rabbits after inoculation with *P. multocida*. Rabbits were inoculated with *P. multocida* as described in Fig. 9. Data points represents means ± SE. ●—● , im; ○---○, in; Δ---Δ, sc. *P < 0.05 relative to time 0.

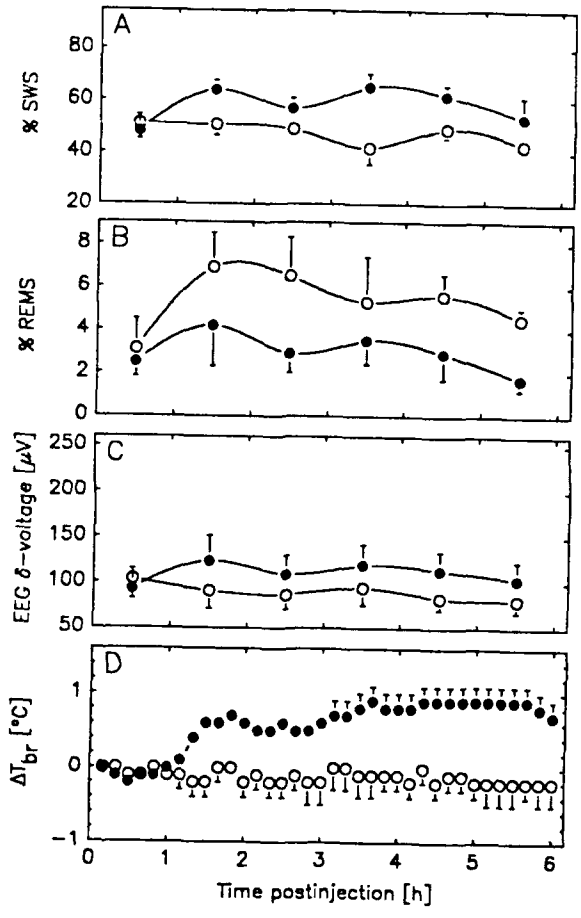

Fig. 16. Effects of intravenous inoculation of 2.4 mg de-O-acetylated staphylococcal cells walls (•) or vehicle (1 ml pyrogen-free saline into same rabbits; ○) on rabbit slow-wave sleep (SWS) (A), rapid-eye-movement sleep (REMS) (B), delta-wave amplitudes (C), and brain temperatures (D). A, B, C: hourly averages for indicated hour. D: changes from temperature (ΔT_{br}) at time 0. A, B, D: n = 8, C: n = 7. Data points are means ± SE.

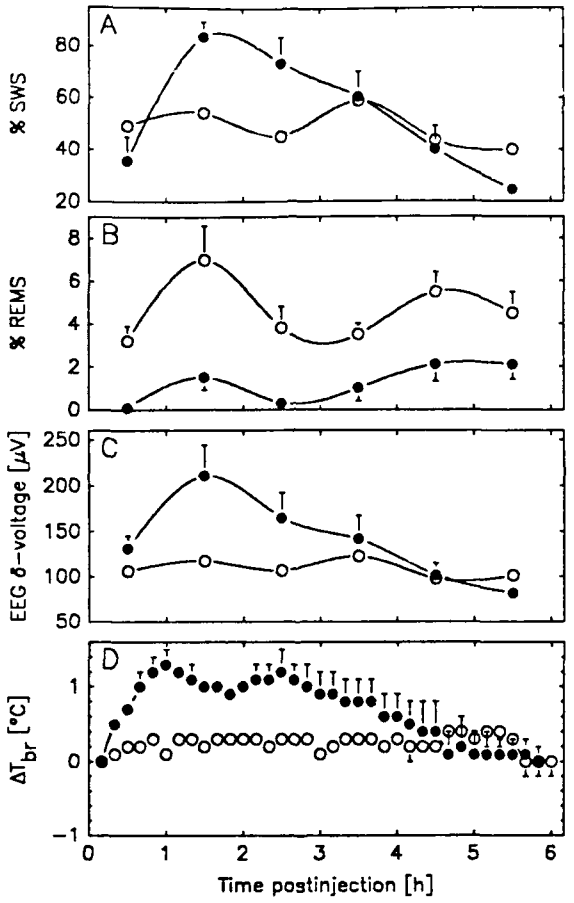

Fig. 17. Effects of intravenous inoculation of 0.5 mg soluble gonococcal peptidoglycan (strain FA19; ●) or vehicle (1 ml pyrogen-free saline into same rabbits; ○) on rabbit slow-wave sleep (SWS) (A), rapid-eye-movement sleep (REMS) (B), delta-wave amplitudes (C), and brain temperatures (ΔT_{br}) (D). A, B, C: hourly averages for indicated hour. D: changes from temperature at time 0; n = 4. Data points are means ± SE.

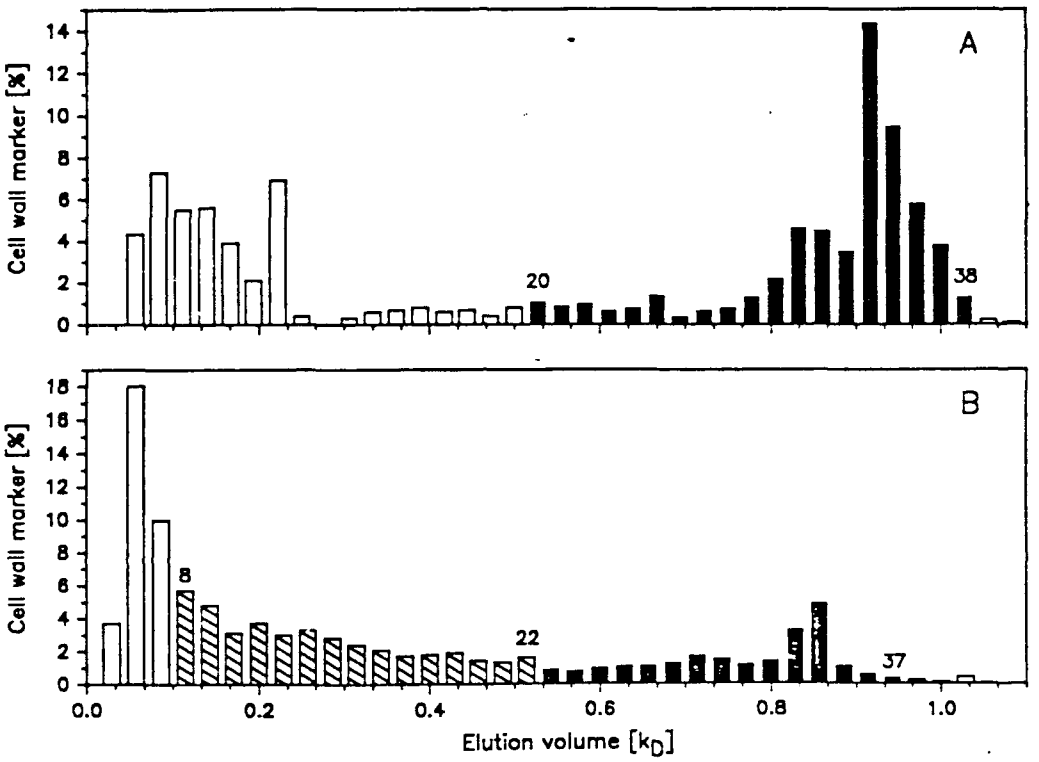

Fig. 18. Separation of soluble fragments of *Staphylococcus aureus* cell walls by gel filtration chromatography (on TSK HW-40, separation range 100 - 10000 D for proteins). A: viable bacteria were digested by macrophages, B: isolated and de-O-acetylated cell walls were degraded by lysozyme. Soluble digestion products were applied to the column. The amount of radioactive cell wall marker (% of total applied) is plotted against elution volume (k_D = $(V_e-V_0)/V_i-V_0$; V_e, elution volume of sample, V_0, total permeation volume; V_i, exclusion volume; V_0 and V_i were determined with NaCl and Blue Dextran 2000, respectively). Fractions were pooled and tested for biological activity as indicated in Table 11 and by marked columns in the figure.

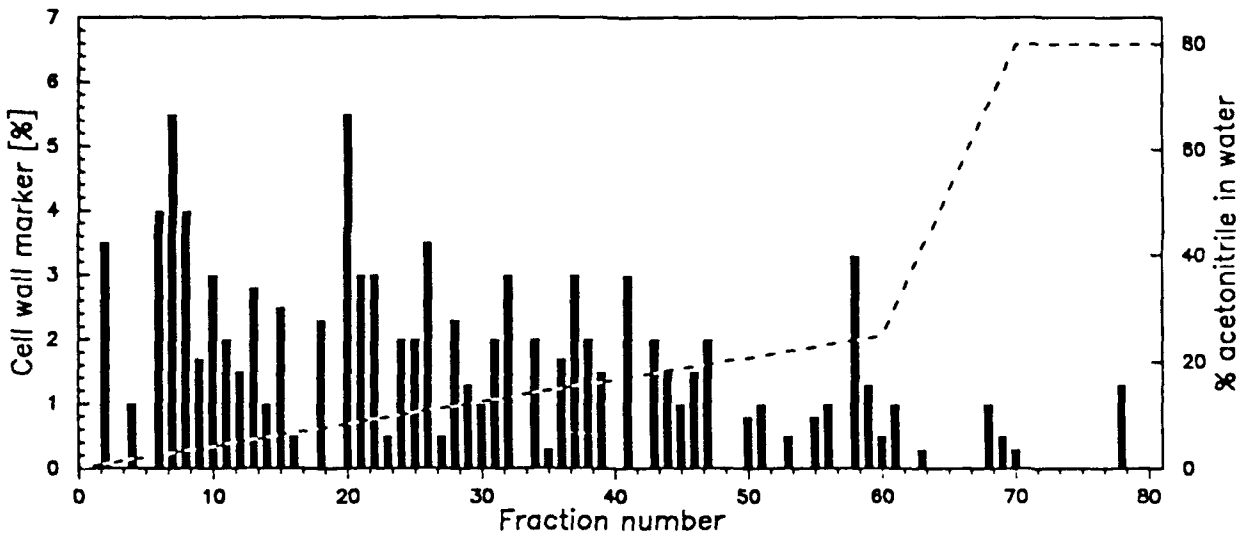

Fig. 19. Separation of *Staphylococcus aureus* cell wall fragments (combined fractions from gel filtration, compare Figure 18A) on C_{18} reversed phase HPLC. Amount of radioactive cell wall marker (% of total applied) is plotted against fraction number. Fractions (0.5 ml each) were pooled as indicated in Table 12 and tested for biological activity.

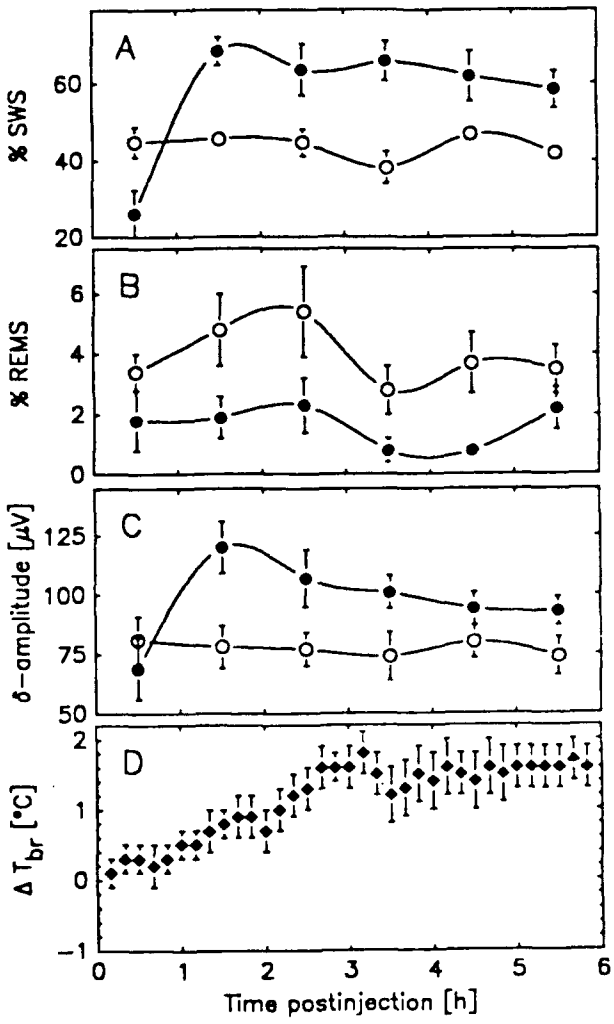

Fig. 20. Effects of intracerebroventricular injection of 350 pmol of pooled fractions 20-38 of macrophage supernatant after separation by gel filtration (compare Figure 18, Table 11) in 25 µl artificial CSF (filled circles) or artificial CSF alone (open circles) on rabbit slow wave sleep (A), rapid-eye-movement sleep (B), d-wave amplitudes (C), and brain temperatures (D). A, B, C: hourly averages for indicated hour. D: average differences experiment - control at indicated time (filled diamonds). A, B, C: n = 8; D: n = 6. Data points are means ± SEM.

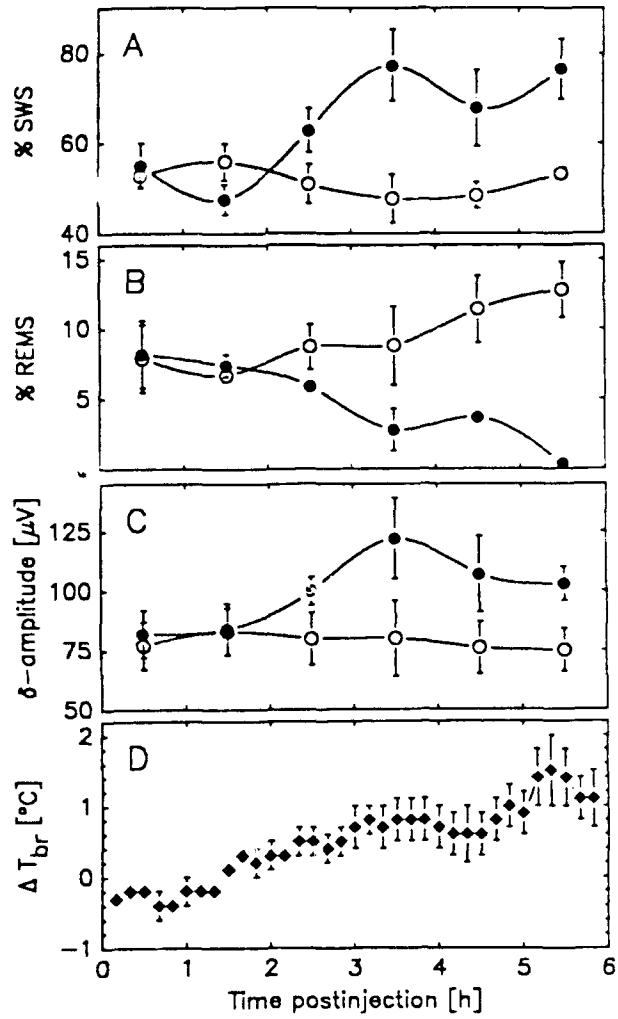

Fig. 21. Effects of intracerebroventricular injection of 10 pmol of pooled HPLC fractions 24-26 (compare Figure 19, Table 12) in 25 µl artificial CSF (filled circles) or artificial CSF alone (open circles) on rabbit slow wave sleep (A), rapid-eye-movement sleep (B), delta wave amplitudes (C), and brain temperatures (D, filled diamonds). A, B, C: hourly averages for indicated hour. D: average differences experiment - control at indicated time. n = 4. Data points are means ± SEM.

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